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The American University in Cairo  
School of Sciences and Engineering

**Characterization of the Bacterial Gut Fauna of *Phlebotomus papatasi*: Culture Dependent vs. Culture Independent Techniques**

By

A Thesis Submitted by  
**Nada Mohamad El-Ayouty**

to the Biotechnology Program

June 9<sup>th</sup>, 2011

in partial fulfillment of the requirements for the degree of

**Master of Science in Biotechnology**

has been approved by

**Dr. Rania Siam (Co-Advisor)**  
Associate Professor and Director of Biotechnology Program, AUC



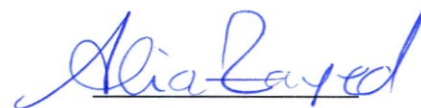
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School of Sciences and Engineering

**CHARACTERIZATION OF THE BACTERIAL GUT FAUNA OF *PHLEBOTOMUS*  
*PAPATASI* :**

**CULTURE DEPENDENT VS. CULTURE INDEPENDENT TECHNIQUES**

A Thesis Submitted to  
The Biotechnology Graduate Program  
in partial fulfilment of the requirements for  
the degree of Master of Science

By:

Nada Mohamad El Ayouty

Under the supervision of:  
Jeffrey T. Villinski, PhD. (NAMRU-3)  
Rania Siam, MD., PhD. (AUC)

June 2011

## ABSTRACT

Characterizing the gut bacteria of sand flies is essential not only to identify their influence on host biology, but also to investigate any potential impact on the establishment and development of *Leishmania* infection, which occurs in the vectors' gut. Gut bacteria may also provide a new avenue to arthropod-borne disease control if a bacterial species typically found in the sand fly can be genetically modified to produce anti-parasitic molecules, thereby producing a vector refractory to disease transmission. This study characterized the gut bacterial community of laboratory reared *Phlebotomus papatasi*, using both a culture based approach and a culture independent approach involving analysis of a 16S ribosomal RNA (rRNA) gene sequence library. The guts of several field flies were also included in the analysis.

These experiments identified 26 species of bacteria in sand fly guts, affiliated with four bacterial phyla: Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes. Laboratory reared flies were predominated by *Leifsonia* spp., regardless of the method of analysis used. Interestingly, *Leifsonia* has not been previously reported in the gut of sand flies. Moreover, bacteria of the genera *Ochrobactrum*, *Stenotrophomonas* and *Bacillus*, previously reported in sand flies from different geographical locations, were also recovered in this study, suggesting the presence of obligate fly-bacterial associations. Bacteria identified that are affiliated with the genera *Achromobacter*, *Wolbachia*, *Leifsonia* and *Bacillus* may be particularly significant due to their ability to deliver transgenes as shown in previous studies. Consequently, they could be considered in the control of *Leishmania* via paratransgenesis.

Results from these experiments support the premise that culture independent approaches are generally more efficient for characterizing bacterial communities. Both the type and diversity of bacteria identified in this study strongly emphasize the significance of the bacteria inhabiting the gut of sand flies. These findings underscore the value of further research aimed at clearly understanding the role of specific bacterial species, and identifying ways in which they may modulate the functions of the vector, the life cycle of the parasite or even disease establishment in hosts.

## TABLE OF CONTENTS

List of Figures.....	v
List of Tables.....	vi
Introduction	
A. Overview	
1. <i>Phlebotomus papatasi</i> sand flies.....	1
2. <i>Leishmania</i> parasites.....	1
3. Prevalence of leishmaniasis.....	2
4. Prevention and treatment.....	2
5. Need for innovative control measures.....	3
B. Gut Bacteria of Hematophagous Vectors	
1. Highlights onto origin and types.....	4
2. Host related functions.....	5
3. Effects on parasite establishment.....	5
C. Gut Bacteria of Sand Flies	
1. Current standing.....	7
2. Limitations and prospects .....	10
3. Goal of this study .....	10
Materials & Methods	
A. Materials	
1. Sand flies.....	11
2. Tools for culture independent analysis.....	11
3. Tools for culture dependent analysis.....	11
4. Computational tools.....	11
B. Methods	
1. Gut extraction.....	12
2. Culture independent analysis.....	12
3. Culture dependent analysis.....	13
4. Computational procedures.....	15
Results	
A. Gut bacteria recovered from laboratory reared <i>P. papatasi</i> using culture dependent analysis.....	16
B. Gut bacteria recovered from field-caught sand flies using culture dependent analysis.....	16
C. Gut bacteria recovered from laboratory reared <i>P. papatasi</i> using culture independent analysis.....	17
D. Comparison between bacterial phyla detected using culture dependent and culture independent methods.....	17
E. Comparison of bacterial phyla recovered from the gut of laboratory reared and field caught sand flies.....	18
Discussion	
A. Design of the study.....	19

B. Comparing bacterial assemblages identified using culture dependent and independent techniques.....	20
C. Gut fauna of field caught vs. lab reared <i>P. papatasi</i> .....	21
D. Prevalent bacteria in sand fly gut: types and potential roles	
1. Actinobacteria.....	22
2. Alphaproteobacteria.....	23
3. Betaproteobacteria.....	27
4. Gammaproteobacteria.....	28
5. Bacilli.....	29
Conclusion.....	30
References.....	44

## LIST OF FIGURES

Figure 1: Distribution of visceral leishmaniasis, worldwide, 2009.....	32
Figure 2: Distribution of cutaneous leishmaniasis, worldwide, 2009.....	32
Figure 3: Phylogenetic affiliations of the bacteria isolated from laboratory reared <i>P.papatasi</i> using culture dependent technique.....	35
Figure 4: Phylogenetic affiliations of the bacteria isolated from field caught sand flies using culture dependent techniques.....	37
Figure 5: Phylogenetic affiliations of the bacteria isolated from laboratory reared <i>P.papatasi</i> using culture independent techniques.....	39
Figure 6: Variation in the bacterial phyla detected by culture dependent and culture independent approaches.....	40
Figure 7: Variation within the classes of Proteobacteria detected by both methods.....	41
Figure 8: Orders of Alphaproteobacteria identified using culture independent and culture dependent techniques.....	41
Figure 9: Bacterial phyla from laboratory reared and field caught flies.....	42

## LIST OF TABLES

Table 1: Preliminary assessment of the isolated pure colonies from culture dependent analysis of laboratory reared <i>P. papatasi</i> .....	32
Table 2: Assembly groups from culture dependent analysis of laboratory reared <i>P. papatasi</i> .....	32
Table 3: Diversity of gut bacteria of laboratory reared <i>P. papatasi</i> (Culture Dependent Analysis).....	33
Table 4: Diversity of gut bacteria of field caught sand flies (Culture Dependent Analysis).....	35
Table 5: Diversity of gut bacteria of laboratory reared <i>P. papatasi</i> (Culture Independent Analysis).....	37
Table 6: Bacterial species recovered from the gut of all sand flies investigated in this study using culture dependent and culture independent techniques.....	43



## INTRODUCTION

### Overview

#### ***Phlebotomus papatasi* sand flies**

*Phlebotomus* spp. are one of the large genera of sand flies (Diptera: Phlebotominae) of the family Psychodidae, subfamily Phlebotominae known to be natural vectors for *Leishmania* spp.<sup>1</sup>. Sand flies are small, hairy flies, about 3 mm in length having a wide geographic distribution occupying temperate, tropical and subtropical zones. Although more than 500 species of phlebotomine sand flies have been identified, only about 10% of them have been incriminated as vectors of *Leishmania*<sup>2</sup>. Interestingly, even among this small proportion, a remarkable specificity in the *Leishmania* spp. transmitted by the different sand fly species has been noted. Some sand flies are permissive- capable of harbouring different types of *Leishmania* species- while others are specific vectors- transmitting only a specific species of *Leishmania* and refractory to all other species<sup>1, 2</sup>. *Phlebotomus papatasi* is an example of a specific vector; sustaining the growth and capable of transmitting infections by *L. major* only<sup>3</sup>.

Because of their need for blood meals to lay eggs, only female sand flies are implicated in the transmission of *Leishmania* from infected to healthy hosts. In the sand fly, the life cycle of *Leishmania* occurs entirely within the gut, and commences shortly after the ingestion of a blood meal from an infected host<sup>2</sup>. Amastigotes of *Leishmania* are present in hosts' macrophages circulating with their blood and are therefore picked up by the fly during feeding. In the digestive tract of the sand fly, these amastigotes initiate the life cycle of *Leishmania*, and develop through a number of stages, to infective promastigotes transmitted to new hosts through the bite of the sand fly as it obtains another blood meal<sup>3</sup>. The parasites' ability to attach to the vectors' gut epithelium and to evade expulsion with blood meal remnants has been noted the most influential factor determining vector ability to sustain and transmit the infection<sup>2</sup>.

#### ***Leishmania* parasites**

These pathogenic protozoa belong to the order Kinetoplastida of the Trypanosomatidae family. They exist in two forms; either as extracellular flagellated promastigotes in the gut of their sand fly vectors where they develop and multiply<sup>1</sup>. On transmission to hosts, they exist as obligate intracellular aflagellated amastigotes in phagolysosomal vacuole of hosts' macrophages. The diverse clinical manifestations of leishmaniasis- ranging from asymptomatic infections, cutaneous and mucocutaneous lesions to fatal visceral infections fatal- are believed to be highly influenced by the causative species of *Leishmania*<sup>3</sup>.

Drug resistance is another aspect that demonstrates the exceptional environmental adaptability of the *Leishmania* parasites and poses a serious challenge in the treatment of

cutaneous and visceral leishmaniasis. Acquired resistance to pentavalent antimonials, the cornerstone therapy for leishmaniasis, is driving this therapy to obsolescence in many parts of the world, particularly India<sup>2</sup>. Intrinsic variations in drug sensitivity among *Leishmania* species is another important issue affecting drugs such as azoles and paromycin. Increased drug efflux via over-expression of ABC transporters (ATP-binding cassette) is one of the molecular mechanisms implicated in multi-drug resistance. Other molecular mechanisms involved in the process of self-adaptation to resistance by *Leishmania* parasites require further investigation<sup>2</sup>.

### **Prevalence of leishmaniasis**

In spite of its serious health impact, leishmaniasis remains a neglected disease. Leishmaniasis is endemic in 88 countries, including 72 developing countries. The annual estimation of new cases of leishmaniasis is about 1.6 million; 150,000 of which are cutaneous and 500,000 visceral<sup>4</sup>. Currently, 12 million people worldwide are infected with leishmaniasis; the infections having significantly increased during the last decade and extended to new geographic areas. In fact, this figure is likely underestimated owing to the fact that a considerable number of cases go unreported, as case reporting is mandatory in only 33 out of the 88 countries where leishmaniasis is endemic<sup>4</sup>.

The increased spread of HIV is complicating the problem to a greater extent as leishmaniasis/HIV co infection poses a deadly synergy. Increased urbanization is another serious problem that threatens to alter the epidemiological trend of leishmaniasis from sporadic cases to epidemics that can be devastating<sup>4</sup>.

Prevalence reports show that the more serious visceral leishmaniasis occurs mainly in Brazil, Bangladesh, India, Nepal, Sudan and Ethiopia (as illustrated in figure 1). Peru, Bolivia and Brazil have the greatest incidence of the mucocutaneous form, while cutaneous leishmaniasis is mostly found in Iran, Saudi Arabia, Algeria, Afghanistan, Syria, Sudan Peru and Brazil<sup>4</sup> (as illustrated in figure 2).

### **Prevention and treatment**

Efforts to control the spread of leishmaniasis involve targeting either the sand fly vectors or the parasites or both. Control programs directed against the sand fly vectors include the spraying of chemicals as insecticides and DDT to decrease their number, or the use of physical methods such as nets to trap them. Nevertheless, as with other arthropod born diseases, the vectors may develop resistance to the insecticides and chemicals used<sup>2,4</sup>.

Control methods that target the *Leishmania* parasite include the elimination of animal reservoirs such as rodents and dogs. Drug therapy against *Leishmania* relied for long on the intramuscular administration of pentavalent antimonials, diamidines, and lately on amphotericin B<sup>2</sup>. Besides the inconvenience in the route of administration, these drugs are expensive and highly toxic; necessitating hospitalization during the course of the treatment, which ranges from 28-30 days. However, the greatest problem remains the increasing

resistance to these drugs; owing to irrational use and inefficient patient compliance due to the long duration of therapy and high costs<sup>2</sup>. This is a serious concern due to the lack of effective second line agents that can be relied on in case of complete failure of these drugs. No vaccines exist for prophylaxis against any form of leishmaniasis although attempts for its production are ongoing<sup>5</sup>.

### **Need for innovative control measures**

Besides being inadequate, these control measures have failed to significantly bring down infection rates. In fact, at the time where the parasite is evolving to become more vicious by locating new vectors, expanding its geographical distribution and becoming refractory to control measures and drugs, attempts at combating the disease fail to keep up with the same pace<sup>2</sup>.

The quest for innovative methods capable of eliminating the parasite while causing minimal or no harm to the transmitting vector was behind the emergence of the paratransgenesis lately<sup>6</sup>. Paratransgenesis is an approach that exploits symbiotic gut bacteria of vectors to halt parasite transmission. This approach takes advantage of the fact that endosymbiotic bacteria and the transmitted parasites share a common residence: the vectors' gut. If the former are capable (or are manipulated) to produce antiparasitic molecules, then they would directly eliminate the parasite as soon as it gains access to the vector<sup>7,8</sup>. The advantage of using endosymbiotic bacteria is their natural presence in the vector; hence, they would be of harm only to the parasite, and would evade ecosystem disturbance resulting from vector elimination. Showing promising results with the Chagas disease vector *Rhodnius prolixus*, attempts have been made to extend this technique to other arthropod borne diseases such as malaria, sleeping sickness and leishmaniasis<sup>6,9,10</sup>.

## **Gut Bacteria of Hematophagous Vectors**

The gut of insect vectors is one of the most interesting sites being the initial point of contact between the invading parasites (ingested with the blood meal) and the epithelial surfaces of the vector<sup>8</sup>. The midgut is generally the place where the parasite first attaches to initiate its life cycle in the vector. Interestingly, a sharp decrease in the number of ingested parasites has been noted at this phase<sup>8</sup>. Gut conditions and other factors such as enzymes, lectins, antimicrobial peptides produced by the vectors' defence system, and the vectors' gut fauna are suspected to be responsible for this decrease<sup>8</sup>. Nevertheless, the potential role of gut bacteria as a determinant of the parasite survival and propagation in hematophagous vectors is yet to be fully elucidated. It is believed that the survival and establishment of important human protozoan parasites in insect vectors is highly influenced by bacteria coexisting in the insects' gut<sup>8</sup>.

### **Highlights onto the origin and types**

In spite of remaining for long one of the areas least investigated, interest in the study of the gut fauna of arthropod vectors of parasitic diseases is resurging. Hematophagous vectors studied for the presence of gut fauna include both laboratory reared and wild caught vectors such as mosquitoes- the vectors of malaria, triatomines- responsible for the transmission of Chagas disease, and sand flies- the vectors of *Leishmania*<sup>8</sup>.

For laboratory reared vectors, bacterial gut fauna is believed to be acquired either through contaminated food (sugar solutions, blood meals or faeces), or via transstadial transmission from adults<sup>9,10</sup>. On the other hand, the exact source(s) of the gut bacteria of wild caught vectors and whether they are transient or permanent gut residents is unknown<sup>11,12</sup>.

Interestingly, literature has reported that less than 50% of the wild caught *Anopheles* harbour bacteria. Moreover, a variation was observed in bacterial counts corresponding to the developmental stage and the feeding status of the mosquitoes: dropping between the larval stage and adult emergence and increasing sharply following blood meals<sup>11,12,13,14</sup>. Investigating the gut fauna of wild mosquitoes collected from Africa revealed the presence of bacterial species belonging to eight genera<sup>15</sup>. These results confirmed previous findings of a study conducted a year earlier on wild mosquitoes collected from India<sup>16</sup>, thereby supporting the view that there exists common representatives of gut bacteria among the different species of mosquitoes. It was also evident that overall, only a small percentage of the mosquitoes under investigation harboured gut bacteria, the majority having only one bacterial species per gut<sup>15</sup>.

Regarding the type of bacteria isolated from the gut of hematophagous vectors, a predominance of Gram negative bacteria was observed. Overall, *E. cloaca* was reported to be the bacteria most frequently associated with insect guts, and naturally abundant bacteria belonging to the genera *Stenotrophomonas*, *Serratia*, *Flavimonas*, *Pseudomonas*, *Enterobacter* and *Acinetobacter* were all reported in the gut of many insects<sup>17</sup>. Both field and

laboratory reared mosquitoes of different species including *Aedes triseriatus*, *Culex pipiens* and *Psorophora columbiae* harboured gram negative bacteria such as *Enterobacter agglomerans*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella ozonae*<sup>11,12,13,14</sup>. Other gram negative bacterial species including *Enterococcus faecalis*, *Enterobacter cloacae*, *Rhodococcus rhodnii* and *Serratia marcescens* were also found to be the most frequently occurring residents of the gut of both laboratory reared and wild triatomines- the vectors of Chagas disease<sup>9</sup>.

### Host Related Functions

Symbiont bacteria have been reported to provide benefits to the insect hosts harbouring them. Dillon *et al.* have reported that break down of polysaccharides in the gut of house crickets is done by *Klebsiella*, *Yersenia*, *Bacteroides*, *Fusobacterium* and *Citrobacter*, whereas *Enterobacter* spp. prevent pathogen colonization of silkworm larvae<sup>18</sup>. Moreover, *Pantoea agglomerans*, *Enterobacter* spp. and *Klebsiella* spp. work together in locusts to produce pheromones and compounds of phenolic nature to ward off pathogenic fungi<sup>18</sup>. Apart from these specific functions, symbiotic bacteria play other general roles in their hosts' metabolism such as maintaining the hypertonicity by secretion of amino acids<sup>19</sup>. They are also a source of nitrogen to their hosts owing to their peptidoglycan rich walls<sup>20</sup> and can breakdown uric acid also to provide nitrogen for their hosts as cockroaches -with the help of *Blattabacterium*- and ants- with the help of *Blochmannia*<sup>21</sup>. For some hematophagous vectors such as mosquitoes, the presence of bacteria has been noted to be essential for larval development. Antibiotics added to rearing water were found to hamper larval development, at times stopping it entirely<sup>22</sup>. Accordingly, it has been suggested that bacteria are a source of larval food. Interestingly, several studies have also shown that volatiles released by some bacteria influence mosquitoes' host preferences and locations for oviposition<sup>22</sup>.

### Effect on parasite establishment

Gut microbiota have been reported to influence the establishment of parasites in insect vectors. A number of studies have pointed out that the presence of vector gut organisms adversely affects the development of parasites<sup>11,23</sup>. For example, *Plasmodium falciparum* oocyst development was found to be blocked by ingestion of *S. marcescens* in *Anopheles stephensi*<sup>11,25</sup>. Bacteria as *Serratia* and *Klebsiella* have been noted to be pathogenic to mosquitoes causing an increase in their mortality, while *Enterobacter* spp. on the other hand was found to be harmless to mosquitoes harbouring it<sup>17</sup>. In a similar trend, lysis of the parasite *Trypanosoma cruzi* was induced *in vitro* by high concentrations of *S. marcescens*<sup>9,11,14</sup>. On the contrary, the symbiont *Sodalis glossinidius* favoured the establishment of this parasite in the midgut of tsetse flies<sup>26</sup>.

Although these studies imply an effect of gut microbiota on the establishment of parasite infections in vectors, the exact mechanism of this modulation is widely debated. It is also apparent that this effect is dependent on both the type and concentration of the bacteria

involved. A number of potential mechanisms have been proposed to explain this effect. The first relies on what is known about the midgut as being an immune-reactive organ; proposing that an increased level of bacteria in the gut evokes an immune response through the expression of certain genes and the production of antimicrobial peptides<sup>11,27,28</sup>. Consequently, this leads to a decrease in the number of both the bacteria and of the infecting parasites<sup>11,27,28</sup>. In other cases such as with the tsetse symbiont *S. glossinidius*, it was suggested that this gut bacterium enhances trypanosome development in the fly by producing inhibitory sugars, thereby neutralizing the anti-trypanosomal effect of the midgut lectins<sup>29</sup>.

A number of microbial factors that can be produced by midgut bacteria could also be responsible for an antiparasitic effect in the vectors' gut. Gram negative bacteria such as *S. marcescens*, *S. plymuthica*, *Klebsiella*, *P. aeruginosa* and *Enterobacter* spp. produce the interesting pigment prodigiosin<sup>30</sup>. This pigment was shown to have a lethal effect on *T. cruzi*<sup>9</sup> and *P. falciparum*<sup>31,32,33</sup>. Other microbial factors produced by bacteria found in vectors' midgut and deemed to have an antiparasitic activity include haemolysins<sup>34</sup>, cytotoxic metalloproteases<sup>35</sup>, and antibiotics<sup>36</sup>.

## **Gut Bacteria of Sand Flies**

The gut of sand flies is a location of special significance being the site where *Leishmania*'s entire life cycle takes place; thereby resident gut bacteria could possibly have a role in modulating this development, either enhancing or inhibiting it. It would also be tempting to speculate that the gut fauna may also be responsible in deciding whether a particular sand fly species would be a restricted or a permissive vector, or even a non vector for *Leishmania*. Yet, this relationship can never be deciphered unless the resident gut fauna of the sand flies is characterized. Furthermore, investigating the gut fauna has currently become more demanding than ever to be able to locate novel methods for vector control due to the limitations and failure of many of the methods currently employed.

### **Current standing**

The gut fauna of sand flies in particular has remained for long one of the areas least investigated and was subject to a lot of controversies. As early as 1929, gut sterility was considered a prerequisite for the ability of sand flies to harbour and transmit parasites, and that *Leishmania* was believed to be unable to survive together with gut bacteria<sup>37</sup>. This view was supported by Hertig in the bulletin of the WHO, stating that bacteria do not occur naturally in guts of sand flies, yet may infect sand flies under contaminated laboratory conditions<sup>38</sup>. Kellick Kendrick tried to provide an explanation for these claims proposing that since blood meals acquired by sand flies are generally free of microorganisms and that they also select their sugar meals carefully from uncontaminated sources like aphids and coccoids, sand fly guts are consequently sterile<sup>39</sup>.

Accordingly, much of the research that followed that dealt with the gut of sand flies did not focus on the bacterial fauna of the gut, but primarily targeted *Leishmania* and its interaction and development in the vectors' gut. Even the few studies that attempted to cultivate the gut content of sand flies were done out of concern that gut bacteria- especially the motile forms- could pose an obstacle when looking for flagellate forms of *Leishmania* in the gut<sup>40</sup>. They were also concerned that gut bacteria may interfere with the well being of colony flies, causing their mortality if they increased in number<sup>40</sup>. Another concern was host infections such as pyogenic abscess and other systemic complications they believed gut bacteria may cause if transmitted to the host during the bite<sup>40</sup>. Even when gut bacteria were detected, they were viewed as a sort of 'contamination' that was attributed to conditions of the field, and their occurrence was considered an uncommon phenomenon<sup>41</sup>.

Among the early studies targeting gut fauna was the one conducted by Rajendran *et al.* on 63 wild flies and four laboratory reared flies<sup>40</sup>. They were unable to isolate bacteria from the gut of the majority of flies (68.66%), while the remaining (31.34%) showed a flora of non pathogenic, saprophytic bacteria as *Bacillus* spp., *Serratia* spp., coagulase negative *Staphylococcus* and *Micrococcus* spp. Only four of the wild caught flies harboured

*Escherichia coli*, and all the four laboratory flies investigated were positive for bacterial gut flora<sup>40</sup>.

In 1985, Schlein *et al.* carried out a similar study that targeted sand flies of the Middle East region, specifically the Jordan valley, attempting to culture their gut fauna<sup>41</sup>. The bacterial gut 'contamination' – as they referred to it- that was recorded in 38.4% of the wild flies examined, was attributed to conditions of the field, and was considered an unusual event<sup>41</sup>. In fact, they suggested that sand flies were able to maintain a bacteria-free gut through secretion of an antibacterial compound in the gut that kept the bacterial counts in check, yet–the exact source of which had to be further investigated<sup>41</sup>.

Perhaps one of the important studies conducted to try to shed light on the existence of gut bacteria in sand flies, their prevalence and nature was that conducted by Dillon *et al.*<sup>42</sup>. It investigated the prevalence and size of gut bacteria in wild caught *Phlebotomus papatasi* while simultaneously investigating a sample of laboratory reared flies to stand upon the variations in the prevalence of gut bacteria between both samples<sup>42</sup>. Prevalence of gut bacteria in wild flies from two different regions was 60% and 47% respectively. However, these ratios were deemed by Dillon *et al.* to be underestimated due to limitations in culture conditions that generally favour fast growing organisms such as the Enterobacteriaceae which- in fact- constituted the majority of their findings<sup>42</sup>. Laboratory flies on the other hand contained bacteria at most sampling times, with a fluctuation in the bacterial counts seen during the lifetime of the sand fly<sup>42</sup>. Similar findings were also reported by Volf *et al.* who showed that the highest prevalence was of Gram negative bacteria two days after blood feeding<sup>24</sup>. A decrease was also observed by the fifth day as the blood meal remnants were excreted together with most bacteria<sup>24</sup>. However, both the studies were unable to indicate whether the bacteria found were transient or permanent members of the vectors' gut.

Analysis of the gut bacteria of the wild caught *Lutzomyia longipalpis* in Brazil by Oliveira *et al.* again revealed a predominance of Gram negative non fermenting bacteria among the eight species of bacteria isolated<sup>43</sup>. This study was entirely based on the use of traditional culture methods for bacterial isolation and identification, and the 245 flies included in this study were analysed in pools of 35. The Gram negative non fermenters isolated included *Acinetobacter lwoffii*, *Stenotrophomonas maltophilia*, *Pseudomonas putida* and *Flavimonas orizihabitans*<sup>43</sup>. Gram negative fermenters found were *Enterobacter cloacae* and *Klebsiella ozaenae*, with *Bacillus thuringiensis* and *Staphylococcus* spp. as the only Gram positive species isolated<sup>43</sup>.

The frequent presence of Gram negative rods belonging to the family Enterobacteriaceae was also noted by Volf *et al.* in an important study investigating the prevalence of gut bacteria corresponding to different developmental stages and feeding traits of *Phlebotomus duboscqi*<sup>24</sup>. A non-fermentative, Gram negative bacteria belonging to the genus *Ochrobactrum* was the most prevalent strain isolated, and was named 'strain AK'. The



authors explained its prevalence by proposing that *Ochrobactrum* together with the other bacteria were obtained through the larval food<sup>24</sup>. Yet while the others were unable to survive the mechanical shedding of the gut that occurs prior to adult emergence, *Ochrobactrum* did, and thereby was detected in large numbers. The other bacteria isolated in this study included *Serratia marcescens*, *Stenotrophomonas maltophilia* and *Citrobacter* spp. that were suggested to be acquired most likely from the blood meal<sup>24</sup>.

Among the few studies that incorporated both culture dependant methods and molecular tools to identify sand fly gut bacteria was that of Gouveia *et al.*<sup>17</sup> and Hillesland *et al.*<sup>6</sup>. However, the molecular tools used in both studies were implemented in the identification of bacterial colonies obtained by culturing, thereby limiting the findings to the small proportion of cultivable microbes. The first study investigated gut fauna of Brazilian populations of *Lutzomyia longipalpis* from both *Leishmania* endemic and non endemic areas<sup>17</sup>, and the second explored gut fauna of *Phlebotomus argentipes* from *Leishmania* endemic areas in India<sup>6</sup>. Bacteria identified were again either affiliated to Enterobacteriaceae or non Enterobacteriaceae groups. Enterobacteriaceae groups included *Morganella*, *Escherichia*, *Citrobacter*, *Enterobacter*, *Pantoea*, *Klebsiella*, *Serratia*, and *Weeksellia*. Non Enterobacteriaceae included *Acinetobacter*, *Pseudomonas*, *Burkholderia*, and *Stenotrophomonas*<sup>17</sup>.

A correlation between the type of microbial gut fauna detected and the area inhabited by the sand fly has been referred to by Hillesland *et al.*, where flies collected from the same region harboured almost the same kinds of bacteria<sup>6</sup>. Therefore, it was suggested that gut fauna diversity is more or less a reflection of the environment where the sand fly resides<sup>6</sup>. For example, *Bacillus megaterium* that is present in biofertilizers widely used in the state of Bihar, India, was isolated from the guts of a number of sand flies inhabiting that area. Another example was that of *Brevibacterium linens*, the bacterium used in cheese ripening industry that was also isolated from the gut of sand flies collected from regions known to be involved in dairy preparations<sup>6</sup>. Both these bacteria were proposed as candidates for use in a paratransgenesis model, being already employed in biotechnological operations without concerns about their safety<sup>6</sup>.

### **Limitations and prospects**

It is important to point out that, to date, no symbiotic bacterial association for sand flies have been identified<sup>6</sup>. Except for a recent study suggesting a dose dependent, inhibitory effect of gut bacteria on *Leishmania* promastigotes, the role(s) played by sand fly gut bacteria is still unknown<sup>44</sup>. Despite the fact that the aforementioned studies helped to provide a preliminary idea about the bacterial life in the gut of this vector, yet the true set back is that almost all the studies analysing the sand fly gut for bacterial communities have relied on culture dependent techniques in their analyses. Even the ones that have implemented molecular tools, used these tools only in the identification and analysis of isolated pure

colonies from plate culture, not in the initial isolation of bacteria from the guts. Taking into account the limitations of culture dependent techniques renders these findings incomplete, thereby questioning many of the concepts that prevailed for long based on these limited findings. This drawback was explicitly referred to in a number of these studies by the researchers who concluded that the failure to isolate bacteria from the majority of flies was primarily due to inefficiencies in the methods employed in bacterial isolation and characterization<sup>6,42</sup>.

Fortunately, the toolkit in the isolation and characterization of microorganisms has largely advanced with the progress made in molecular technologies. The use of 16S rRNA primers allows selective amplification of bacterial symbionts without amplification of hosts' DNA<sup>22</sup>, thereby enabling a more comprehensive isolation and characterization of gut fauna of arthropod vectors of parasitic diseases.

### **Goal of this study**

This study was primarily designed to characterize the gut bacteria of laboratory reared *P. papatasi* using both culture dependent and culture independent techniques to achieve three goals. First, to develop a culture independent procedure for isolating bacteria from the gut of sand flies to overcome the limitations of culturing techniques. Secondly, to compare the bacterial communities identified by both methods and to compare that of laboratory reared flies with field caught flies. Finally, to determine if the bacterial gut fauna of sand flies in this region compares with that of other records, in an attempt to locate common gut bacteria that can serve as candidates for a paratransgenesis model to halt the spread of *Leishmania*.

## **MATERIALS & METHODS**

### **Materials**

#### **Sand flies**

Laboratory colonies used in this study consisted of newly emerged, unfed (unexposed to blood or sugar meal), *Phlebotomus papatasi* female sand flies maintained at NAMRU-3 insectaries. Several field flies were obtained from two areas in northern Egypt where *P. papatasi* is prevalent and were included in the study. Seven of these flies were caught at St. Catherine and the remaining four from Firan in August 2009. Sand flies were transported alive to NAMRU-3 and processed upon arrival. For gut extraction, a light microscope was used. Surface decontamination of flies was done by absolute alcohol. Gut extraction was carried out using sterilized slides wiped with absolute alcohol and DNA-Erase<sup>®</sup>, and needles treated in the same way.

#### **Tools for culture independent analysis**

For DNA extraction and processing: QIAamp<sup>®</sup> DNA Mini Kit (Qiagen) for DNA extraction, QIAquick PCR purification kit (Qiagen), universal 16S rRNA PCR primers 27F and 1492R<sup>45</sup>, 9600 Thermocycler, Big dye Terminator kit, DyeEx 2.0 spin kit and ABI PRISM 310 automated DNA sequencer.

For 16S rRNA gene library construction, plasmid extraction and purification: *Escherichia coli* JM109 competent cells, pGEM<sup>®</sup>-T Easy Vector System (Promega), LB-Amp plates and broth, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal), and Quick Lyse Miniprep Kit (Qiagen) and NanoDrop instrument (ThermoScientific).

#### **Tools for culture dependent analysis**

Media used for the isolation of bacteria included: Tryptone Soy Agar (TSA), Brain Heart Infusion Agar (BHI), Blood Agar, MacConkey's Agar, TSA/Blood Agar (TSA supplemented with 5% blood) and sterilized phosphate buffered saline.

For microscopical and biochemical examination: Gram stain, reagents for oxidase and urease tests.

For genomic DNA processing: same tools as the ones used for DNA extraction and processing described above.

#### **Computational tools**

Tools at Ribosomal Database Project (RDP): 16S rRNA pipeline, sequence match and hierarchy browser<sup>46</sup>, Bellerophon Chimera check<sup>47</sup>, Sequencher 4.10.1 software (Gene Codes Corporation), BioEdit<sup>48</sup>, MEGA4<sup>49</sup> and BLAST service at NCBI<sup>50</sup>.

## **Methods**

### **Gut extraction**

Sand flies were killed by chilling at -4°C for 30 minutes. Surface sterilization of the flies was then carried out by dipping each fly in 20µl absolute alcohol for 10 seconds. This procedure was repeated three times for every fly using fresh absolute alcohol each time. This was followed by a final rinsing step in nuclease free water for each fly. Dissection slides and needles were wiped with absolute alcohol and DNA-Erase® prior to the dissection of each fly. Flies were dissected in 10µl of nuclease free water under a light microscope and intact guts were isolated. Due to the inability to culture bacteria from individual guts-due to the small gut volume (less than 0.3 µl<sup>42</sup>) the guts extracted were pooled in 1.5ml microcentrifuge tubes in pools of either five (culture independent analysis) or ten guts (culture dependent analysis). Contents of each tube were homogenized thoroughly with a sterilised pestle to release gut contents, which were immediately either plated on culture media or processed for DNA extraction. The guts taken for plating were homogenized in 70µl of sterilized phosphate buffered saline prior to plating. This was done to suspend the bacteria in the liquid medium thereby enabling their spread on culture media.

To ensure the efficiency of the surface sterilization procedure of the flies and of the utensils used, a few drops of the nuclease free water left on the slides after gut extraction were plated on culture media. This served as a negative control to ensure that the origin of any bacteria obtained at later stages is the insect gut and not the surface of the fly or contaminated utensils.

### **Culture independent analysis**

Forty guts were extracted from sand flies as described above, and divided into 8 pools; each containing 5 guts. DNA was extracted from each pool using QIAamp® DNA Mini Kit (Qiagen) following the 'Tissue Protocol' described by the manufacturer for isolating DNA from tissues.

Adjusting the conditions for PCR amplification of bacterial DNA isolated directly from guts was challenging due to the absence of any protocol in the literature at the time of the analysis describing the conditions required for amplification of bacterial DNA directly from the gut of sand flies. All previous studies investigating sand fly gut bacteria relied on the amplification of bacterial DNA from plate cultures<sup>6,17</sup>. After numerous unsuccessful trials, successful amplification was achieved using the following protocol and conditions for the reaction mixture: 5µl DNA template, 10µl 5X Green Go Taq Buffer, 5µl 25mM MgCl<sub>2</sub>, 2µl 2.5mM dNTP's, 1µl of the forward and reverse primers each, 25.4µl of DNA-free sterilized PCR water and 0.6µl GoTaq flex DNA polymerase (5u/µl). Thermocycler conditions were adjusted to include an initial denaturation step for 12 min at 95°C, followed by 30 cycles of 95°C for 15 seconds, 55°C for 20 seconds and 72°C for 20 seconds, then a final extension

step at 72°C for 30 minutes. Negative controls using DNA free sterilized PCR water were carried out in parallel with all the reactions.

Successful amplification was ascertained by visualization of bands of the correct size under UV following gel electrophoreses using 2% agarose and staining with ethidium bromide. PCR amplifcons were then purified using QIAquick PCR purification kit (Qiagen). Gel electrophoreses of the purified DNA followed to ensure the presence of a sufficient quantity of DNA remaining after purification; reflected by the quality of the bands appearing on the gel.

These amplified purified PCR products comprised a mixture of the 16S rRNA genes from all the bacteria present in the sand fly guts. To be able to study these genes individually to identify each bacterium, a 16S rRNA gene library was constructed. The construction of the library was carried out as follows: 10µl of the purified PCR product from each pool was taken and the total amount of DNA was then measured using NanoDrop to enable accurate calculation of the amount of vector to be used. This was followed by ligation into pGEM-T Vector system (Promega) according to manufacturer's instructions and transformation of *Escherichia coli* JM109 competent cells. Selection of transformants was done using LB-Amp (100µl/ml) plates containing 32 µl X-gal (50mg/ml).

Plasmid DNA was then isolated and purified from the transformants positive for the insert using QuickLyse Miniprep Kit (Qiagen) and subsequently amplified and run on a gel to ensure the presence of the 1.5kb 16S rRNA gene. This was followed by bidirectional sequencing with BigDye reaction mix as per manufacturer's instructions using the plasmid primers T7 and Sp6 that flank the insert DNA in pGEM-T Vector system. Approximately 550-600bp from both ends of the 1500bp 16S gene were obtained.

### **Culture dependent analysis**

A number of preliminary experiments were conducted to select the most appropriate media and conditions for the isolation of the bacterial gut fauna of sand flies. These included the use of a number of non selective media such as TSA, BHI, Blood agar and TSA/Blood agar for initial isolation of bacteria. TSA/Blood agar was chosen as the non selective medium to be used in this study as it has proven upon experimentation to be the medium capable of sustaining the growth of both the largest number and diversity of bacterial strains in comparison with others.

Incubation times were also varied in initial experimentation between 25°C and 37°C for 24 or 48 hrs to decide on the optimal conditions favouring the growth of gut bacteria of sand flies. Incubation at 25°C for 48 hrs was found upon initial experimentation to be optimal, therefore was used throughout this study. All bacteria were isolated under aerobic conditions.

Six pools containing 10 guts each were plated on TSA/Blood agar- immediately after gut extraction without growing first in simple broth for enrichment. This was done to exclude the discrepancies that sometimes arise due to the bias of enrichment techniques towards fast growing organisms. After incubation, colonies appearing on TSA/Blood agar were initially counted then characterized morphologically. Pure colonies of each type were then isolated on fresh media, and freezer-stocks were prepared and stored at -70°C. Preliminary assessment of isolated pure colonies was carried out by testing their Gram reaction, followed by culturing them on MacConkey agar and testing their oxidase and urease reactions.

Finally, to identify the bacteria, analysis of genomic DNA was carried out on pure colonies of all isolates. This included four main steps: DNA extraction from colonies, amplification using universal 16S primers, purification and gel electrophoresis of the PCR product, and finally sequencing of amplicons. Procedures and conditions of these reactions are described below:

DNA extraction: DNA was extracted using QIAamp® DNA Mini Kit from Qiagen following the manufacturer's instructions describing the isolation of genomic DNA from bacterial plate cultures.

PCR amplification: Reaction mixtures for the amplification included 5µl DNA template, 10µl 5X Green Go Taq Buffer, 5µl 25mM MgCl<sub>2</sub>, 2µl 2.5mM dNTP's, 1µl of the 8-27F and 1492-1510R primers each, 25.4µl of DNA-free sterilized PCR water and 0.6µl GoTaq flex DNA polymerase (5u/µl). Thermocycler conditions were adjusted to include an initial denaturation step for 12 min at 95°C, followed by 30 cycles of 95°C for 15 seconds, 55°C for 20 seconds and 72°C for 20 seconds, then a final extension step at 72°C for 30 minutes. Negative controls using DNA free sterilized PCR water were carried out in parallel with all the reactions.

PCR product purification and visualization: the PCR product obtained was purified using QIAquick PCR purification kit (Qiagen), together with negative controls. The purified amplicons and the negative controls were then visualized under UV light, after running on a 2% agarose gel stained with ethidium bromide.

Sequencing reactions were carried out on the purified amplicons using the BigDye reaction mix following manufacturer's instructions. Finally, bidirectional sequencing was done using the same primers used in amplification. Approximately 550-600bp from both ends of the 1500bp 16S gene were obtained.

The analysis of the 11 wild flies included in this study was culture-dependent, with the difference being that individual guts were plated this time. This was due to the availability- in fact abundance- of colonies obtained on plating of single guts on culture media. Owing to the large number of colonies obtained per gut and their great similarity, isolation of pure colonies was not feasible. Therefore, the total genomic DNA from each plate was extracted,

amplified as described above, and the PCR products purified. These amplified purified PCR products comprised a mixture of the 16S rRNA genes from all the bacteria present in the wild fly guts. To be able to study these genes individually to identify each bacterium, a 16S rRNA gene library was constructed. This is the second library constructed in this study, and it was constructed and processed exactly like the former.

### **Computational procedures**

DNA sequences processing: Sequence chromatograms were visually examined then edited by BioEdit<sup>48</sup> and checked for the presence of chimeras using Bellerophon chimera check<sup>47</sup>. Poor quality sequences and chimeric sequences were excluded from further analysis. Sequences were then uploaded to RDP and compared with the database at RDP using the sequence match function<sup>46</sup>, and with the GenBank database using BLAST (blastn suite)<sup>50</sup>. Sample sequences with similarity of  $\geq 97\%$  to the ones at GenBank were considered to be an exact species match, while those with  $\geq 95\%$  but  $< 97\%$  similarity scores, were regarded as novel species within the genus with the highest match at time of analysis (Feb 2011)<sup>51</sup>.

Raw sequences obtained from the clone libraries were downloaded to the RDP pipeline where they were subjected to base calling by Phred, then vector removal and quality trimming by Lucy<sup>46</sup>. The presence of chimeric sequences was detected by Bellerophon chimera check<sup>47</sup>, and all poor quality sequences and chimeras were removed. Approximately 550-600bp from both ends of the 1500bp 16S rRNA gene were obtained for every clone. The clones of each library were then assembled using Sequencher 4.10.1 software, placing sequences with at least 99% similarity within the same group. The sequences were then identified by comparison with the databases as described above. Good's method was used to calculate the coverage for each library, using the formula  $[1-(n/N)]*100$  where  $n$  is the number of molecular species represented by one clone and  $N$  is the total number of sequences<sup>52</sup>.

The resulting sequences and their homologs- which were downloaded from RDP<sup>46</sup> and GenBank<sup>50</sup>- were used for phylogenetic analysis. Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 was used for aligning these sequences and for the creation of neighbour-joining phylogenetic trees using Kimura 2 evolutionary model<sup>49</sup>. Trees were evaluated by bootstrap analysis (1000).

The trees were created based on only the 500bp forward sequences the V1-V3 regions of the 16S rRNA gene (approximately 484bp) as they offer the greatest phylogenetic resolution than the other regions being more divergent<sup>53</sup>. High resolution is particularly essential when analysing microbial communities in specialized habitats including waste-water treatment reactors, intestinal tracts and the rumens<sup>53</sup>.

## RESULTS

### **Gut bacteria recovered from laboratory reared *P. papatasi* using culture dependent analysis**

A total of 60 guts were cultured in pools of 10 (10\*6). A total of 14 colonies were obtained from these 6 pools. The initial morphological characterization and preliminary tests carried out on the isolated pure colonies from each pool are shown in table 1. It was evident that both the number and nature of bacterial isolates varied between the pools.

Amplification of the 16S rRNA from the isolated pure colonies followed by sequencing revealed the presence of common sequences i.e. sequences occurring in more than one pool. Therefore, all sequences were assembled and identical sequences were placed within the same group (as illustrated in table 2) before comparison with the database for identification.

Blastn of the 16S rRNA gene sequences and their classification according to the closest match in the GenBank revealed the presence of seven isolates belonging to the phylum Actinobacteria. Among these, the genus *Leifsonia* predominated with six isolates, in addition to a single isolate belonging to the genus *Arthrobacter*. The next most abundant phylum was Proteobacteria having 6 isolates comprising mainly of the Alphaproteobacterium *Ochrobactrum intermedium*, whereas Gammaproteobacteria was represented by a single isolate of *Stenotrophomonas maltophilia*. Finally, the last isolate recovered using this approach was affiliated with the phylum Bacteroidetes, however, it was unidentified at either the species or genus levels. Table 3 describes the classification of all isolates according to their 16S rRNA gene sequences providing a graphical representation of the relative abundance of the identified phyla. Phylogenetic affiliations of the identified bacteria and their relationship with relative species are displayed in figure 3.

### **Gut bacteria recovered from field-caught sand flies using culture dependent analysis**

Eleven field caught flies were included in this analysis. As plating of individual guts of wild flies resulted in dense growth on culture plates making the isolation and purification of pure colonies unfeasible, a clone library were constructed from the collective DNA extracted from all plates.

A total of 137 clones were positive for the insert (the 1500bp 16S rRNA gene) and all were subjected to bidirectional sequencing. Only the forward sequences (500bp) were used in the analysis and they amounted to 128 after the exclusion of chimeric and low quality sequences. The coverage calculated for the 128 clones using Good's method was 95.3%.

Table 4 illustrates the affiliation of the bacterial isolates obtained from guts of field flies after comparison of their 16S rRNA gene sequences with GenBank. Of the 128 bacterial clones analysed, about two thirds (80 isolates) were Gammaproteobacteria almost entirely



comprising of *Rahnella* species. The remaining 48 isolates belonged to family Bacillaceae of the bacterial phylum Firmicutes. Phylogenetic affiliation of these isolates is illustrated in figure 4.

#### **Gut bacteria recovered from laboratory reared *P. papatasi* using culture independent analysis**

Forty guts of laboratory reared *P. papatasi* were used in the culture independent analysis. From the library constructed, 111 clones were found positive for the insert (the 1500bp 16S rRNA gene) and all were bidirectionally sequenced. Only forward sequences (500bp) were used in the analysis, and they amounted to 80 after the exclusion of chimeric and low quality sequences. The coverage calculated for the 80 clones was 96.3%.

Table 5 illustrates the identity of these bacterial isolates after comparison of their 16S rRNA sequences with GenBank and their phylogenetic affiliations is depicted in figure 5. Fifty one isolates were identified as the Actinobacterium *Leifsonia*. Next in abundance in the gut of laboratory flies analysed using culture independent techniques was the *Wolbachia* spp. (represented by 14 isolates) followed by *Achromobacter xylosoxidans* (10 isolates). Both the aforementioned species are Proteobacteria that were not detected by culture dependent techniques. *Ochrobactrum* spp. was another Proteobacterium detected in both analyses although it occurred here at a lower percentage (6.17%) compared to the culture dependent analysis (35.7%).

#### **Comparison between bacterial phyla detected using culture dependent and culture independent methods**

In this comparison, field flies were excluded and only the gut bacteria of the 100 laboratory reared flies analysed were taken into consideration. This was done so that the only variable assessed would be the technique used (culture dependent vs. culture independent) excluding the influence that the place of collection of flies could possibly have. The variation in the phyla detected by culture dependent and culture independent techniques is illustrated in figure 6. As displayed, the phyla Actinobacteria and Proteobacteria were detected by both methods, although a variation is seen in the percentage of bacterial isolates detected within these two phyla. Moreover, the phylum Bacteroidetes identified by the culture dependent analysis did not appear in the culture independent analysis.

Nevertheless, despite the fact that the first glance at the figure might imply that culture dependent analysis is more efficient than its counterpart in elucidating the bacterial species inhabiting the sand fly gut, an in depth analysis of the results proves otherwise. This is illustrated in the figures 7 and 8. One such example is of the Phylum Proteobacteria. Although being detected by both methods, different classes were identified by each of the different methods as illustrated in figure 7. Furthermore, even within the classes identified by

both methods such as Alphaproteobacteria, there was also a variation seen between the ability of both methods to detect different orders within this class as illustrated in figure 8.

### **Comparison of bacterial phyla recovered from the gut of laboratory reared and field caught sand flies**

Figure 9 illustrates the bacterial phyla isolated from laboratory reared and field flies by culture dependent techniques. Among the other phyla detected, phylum Proteobacteria was found to be the common bacterial phyla retrieved from the gut of flies from the two locations.

Finally, a comprehensive overview of all the bacterial species recovered from the gut of all sand flies investigated in this study using culture dependent and culture independent techniques is illustrated in table 6.

## DISCUSSION

The scarcity of studies investigating the presence and importance of the bacterial gut fauna of sand flies is not the true setback for this area of research. Even the few studies investigating bacterial community assemblages in sand flies guts have a critical shortcoming: they rely principally on culture dependent techniques. This approach may profoundly underestimate the true diversity of the bacterial populations in environmental samples<sup>54</sup>. Even the studies that have used molecular tools in their analyses have characterized genomic DNA obtained from plate cultures, rather than DNA obtained directly from the gut, thereby restricting their findings to a small sector of cultivable microbes.

This study was designed to characterize the gut bacteria of *Phlebotomus papatasi* using both culture dependent and culture independent techniques. In doing so, the study had three specific goals. First, to develop a culture independent procedure for isolating bacteria from the gut of sand flies, thereby avoiding the limitations of culture dependent approaches. Secondly, to compare the bacterial communities identified by the two methods and determine how the fauna of field caught flies differs from laboratory reared colonies. Lastly, to determine how the bacterial gut fauna of sand flies in this region compares with other records, and attempt to locate a bacterium common to all sand flies which could serve as a candidate for a paratransgenesis model to halt the spread of *Leishmania*.

### Design of the study

A total of 111 female sand flies were analysed in this study. Of these, 100 were laboratory reared *P. papatasi*, 60 of which were taken for culture dependent analysis, and the remaining 40 analysed using culture independent techniques. The 11 field flies investigated were caught from Central Sinai, an area with high prevalence of *P. papatasi*. Being the predominant vector for leishmaniasis in Egypt, *P. papatasi* was the sand fly species selected for this study.

Only newly emerged, unfed flies, were collected from laboratory colonies, to ensure that any bacterium isolated from the gut was not obtained from an exogenous source such as blood or sugar meals taken by the fly. Having recently emerged, any bacteria isolated from these flies are likely to have undergone transstadial transmission (from larvae to the adult flies) suggesting a role in the normal biology of the sand fly. Male flies were not included in this study as *Leishmania* is only transmitted by female sand flies. Some studies have used whole crushed flies to investigate gut fauna; however, it is unclear if the gut is the actual source of the bacteria detected. To avoid this concern, this study analysed intact guts microdissected from the flies.

The low density of bacterial population inhabiting the sand fly gut and this organ's small volume necessitated the pooling of dissected guts prior to analysis. Even with pooling, a relatively small number of bacteria were obtained on culture dependent analysis of

laboratory flies. Plating a pool of 10 guts would often yield just one or two colonies, at the most five colonies, and the net yield from all 60 guts investigated this way was only 14 colonies.

The culture independent characterization posed alternative challenges. Amplification of bacterial DNA using universal 16S rRNA primers directly from the sand fly gut tissue has not been previously reported to the time of this study, therefore experimentation was required to develop and optimize an amplification protocol. Even when successfully obtained, these PCR products comprised a complex mixture of 16S rRNA genes from all the bacteria present in the sand fly guts. To isolate individual 16S rRNA genes for sequencing, a clone library had to be constructed. This was the first library constructed in this study, and the second one was constructed for field caught flies analysed culture dependently.

An approximately 1500bp product was amplified from the 16S rRNA gene but only 500bp, constituting the V1-V3 region of the gene, was used for sequence analysis and the construction of phylogenetic trees. This region has been reported to be a more divergent region in this gene offering a greater a phylogenetic resolution essential when analysing communities in specialized environments such as the gut<sup>53</sup>.

### **Comparing bacterial assemblages identified using culture dependent and independent techniques**

A total of 15 bacterial species were identified from the gut of the 100 laboratory flies analysed in this study, 10 of which were recovered by culture independent techniques and the remaining five were obtained by culturing. Gut analysis of the 11 field caught flies using culture dependent methods resulted in the recovery of 11 bacterial species.

Bacterial isolates affiliated with the genus *Leifsonia* were the most abundant in laboratory reared flies; constituting almost 50% of the isolates recovered from by culture dependent techniques and 64% of the guts analysed culture independently. The next most abundant phylum in the gut of laboratory reared flies was Proteobacteria; 43% of the isolates analysed culture dependently and 36% of those recovered using culture independent techniques.

Nevertheless, there were several distinctions to be noted in the findings of both methods. Although fewer guts were analysed using the culture independent approach, a greater diversity of bacteria was identified. Culture dependent techniques failed to detect entire classes such as Betaproteobacteria to which *Achromobacter* species is affiliated. At other times, an entire order such as Rickettsiales of the class Alphaproteobacteria was not detected despite detecting other bacteria belonging to the order Rhizobiales of the same class. The aforementioned bacteria that were not detected by the culture dependent technique are all aerobic; therefore the failure to detect them reflects and supports the fact that culture based techniques are limited in their ability to reveal the true microbial diversity.

Even for the bacterial genera detected by both methods, distinctions were apparent in the ability of each method to reflect the diversity at the species level. For example, in the genus *Leifsonia*, a single species was obtained using culture dependent analysis yet several species were isolated using culture independent analysis.

However, three isolates detected on culturing: the unidentified colony belonging to the phylum Bacteroidetes, and the two belonging to the genera *Arthrobacter* and *Stenotrophomonas*, failed to have counterparts in the culture independent analysis. This could be either due to the scarcity of these bacteria in the gut of sand flies; therefore they were not present in the guts taken for culture independent analysis. A second possible explanation is that they were present, and there was a failure in their amplification and identification using molecular processes. Failure of culture independent procedures to recover all bacterial species in certain samples has been previously reported and attributed to possible interference by remnants of midgut cells in the PCR or the competition of different bacterial DNA during the amplification process favouring the highly abundant ones<sup>22</sup>.

#### **Gut fauna of field caught vs. laboratory reared *P.papatasi***

Because the bacterial fauna of field flies was only recovered by culture dependent techniques, bacterial species isolated cannot be taken to reflect the entire bacterial gut fauna of field flies. Therefore, the comparison made in this study was only done to give a preliminary idea about the possible existence of common phyla among field and laboratory reared sand flies. This could prospectively help in the identification of either bacterial symbiotic associations with sand flies, or the location of gut bacterium that can be used in paratransgenesis.

Despite having undergone only a culture dependent analysis, two significant results were revealed on analysis of the gut fauna of field caught flies. The first is that the gut bacteria of field flies apparently outnumber by far that of their laboratory reared counterpart. This was evident by the fact that plating single guts on culture media did not only yield visible colonies, but a large number of them that were, in fact, too numerous to count or to isolate separately. This necessitated the construction of a clone library from the total genomic DNA isolated from plate colonies of field caught flies to enable their identification. Finding a large number of gut bacteria in field flies was not unexpected owing to their presence in an open environment, and their possible acquisition of contaminated meals (either blood or sugar or both) from various sources.

The second interesting finding that was revealed is that their gut fauna apparently did not resemble to a large extent that detected for laboratory reared flies. Although it shared with laboratory flies the presence of Proteobacteria, a different genera was found in abundance here: the genus *Rahnella*. It constituted almost 63% of the isolates, and the remaining isolates belonged to the Bacilli class. It was also notable that unlike *Rahnella* where almost all the species belonged to a single species, there was a variety in the *Bacillus* spp. identified.

## Prevalent bacteria in sand fly gut: types and potential roles

Findings of this study revealed the occurrence of several bacterial genera with different abundance in the sand fly gut. To stand upon the significance of these findings and the potential role(s) of these bacteria in contributing to the pathogenesis of leishmaniasis or alternatively as prospective tools for vector control and disease elimination, it would be necessary to view these bacteria in context of their place in nature: where have they been found and what have they been known to do.

### Actinobacteria

Beginning with the most prevalent bacterial genus in the gut of laboratory reared *P. papatasi*- the genus *Leifsonia* – it would be important to mention its affiliation with the phylum Actinobacteria. In nature, species of Actinobacteria are highly diverse both physiologically and morphologically. This enables them to play highly divergent roles in the environment, industry and also to act as prominent human, plants and animal pathogens<sup>55</sup>. The pathogenic *Mycobacterium* and *Corynebacterium*, in addition to *Bifidobacterium* the gut commensal, and the antibiotic producing *Streptomyces* all belong to this phylum<sup>55</sup>.

The genus *Leifsonia* contains species isolated from diverse ecological niches; including soil (*L. shinshuensis* and *L. naganoensis*), distilled water (*L. aquatica*), Antarctic ponds (*L. aurea* and *L. rubra*) and plants (*L. xyli* subsp. *xyli*, and *L. xyli* subsp. *cynodontis* and *L. poae*)<sup>56</sup>. Its occurrence in insect guts has been reported recently by Morales-Jimenez, isolating *L. shinshuensis* from the gut of the red turpentine beetle<sup>57</sup>.

*Leifsonia* has not been previously reported in the gut of sand flies. Species of *Leifsonia* identified in this study include *L. xyli* strain X11 as the most abundant species, followed by *L. shinshuensis*. The species *L. xyli* contains two subspecies; subsp. *xyli* (*Lxx*) and *cynodontis* (*Lxc*)<sup>58</sup>. To date, sugar cane is known to be the unique natural host for *Lxx*, which is found in association with the pits and lumen of its xylem-vessels. *Lxx* is widely known for the great economical losses it causes being the causative bacterial pathogen of the major disease afflicting sugar cane worldwide: the ratoon stunting disease<sup>59,60</sup>.

*Leifsonia* is not considered a soil-borne pathogen. It is believed that infection of sugarcane with *Leifsonia* occurs mechanically through tools and machinery contaminated with sap from infected plants<sup>59</sup>. No natural sources of infection have been identified so far, although investigations to locate potential modes of infection, other hosts or insect vectors are yet to be conducted. Owing to the difficulty of growing this fastidious, xylem-limited, bacterial pathogen *in vitro*, there was a lack of information about its pathogenicity and biology for a long time<sup>59</sup>.

Nevertheless, the recent sequencing of its genome revealed several interesting facts about this organism<sup>59</sup>. The genome which is believed to have been progressively decaying as

a result of converting to a restricted lifestyle, was found to contain a large number of pseudogenes- outnumbering all bacterial plant pathogens that have been sequenced. Moreover, among the mobile genetic elements identified were 5 distinct insertion sequence (IS) families responsible for 50 insertions all over the chromosome that are in close proximity to genes coding for known functions such as transporters and regulatory elements<sup>59</sup>.

Comparing it to *Xylella fastidiosa*, another xylem pathogen with a similar genome size, a number of striking differences appear. *Leifsonia xyli*'s contains genes capable of metabolizing and transporting a large number of sugars, resembling those associated with free living organisms, unexpected to be found in an organism associated with carbon-poor xylem cells as itself<sup>60</sup>. Moreover, *L. xyli* subsp. *xyli* contain many genes encoding proteins involved in interactions with living cells, despite the fact that xylem cells are considered dead cells<sup>60</sup>. Furthermore, it was found to encode products protecting it from reactive oxygen species – perhaps in response to host defence systems- in addition to encoding for a multi-drug efflux pump<sup>56</sup>.

To explain these findings, it was suggested that *Leifsonia* could possibly be inhabiting other niches that are yet to be discovered<sup>60</sup>. Finding *Leifsonia* in abundance in the gut of newly emerged sand flies questions the possibility of whether the gut of sand flies is another niche occupied by this organism. Alternatively, another suggestion provided by researchers to explain these findings is that *L. xyli* may have been a free living organism some time in history and then began adapting to a restricted lifestyle in the xylem tissues after the loss of important functional genes<sup>60</sup>.

On the other hand, (*Lxc*) the second subspecies of *Leifsonia xyli* is very different. *Lxc* showed tremendous potential in being used to express useful foreign genes in plants through its insertion sequences<sup>58</sup>. Isolated originally from the xylem of Berumda grass, *Lxc* does not cause stunting symptoms as *Lxx* and was also found to grow in high titres in the xylem of agriculturally important crops such as corn, rice and sugar cane on artificial inoculation<sup>58</sup>. In fact, it was found to increase the growth of some strains of rice, the fact that triggered research into using *Lxc* as carrier to express beneficial genes in crops of interest as rice which was successfully achieved<sup>61</sup>. Among the promising insertion sequences found and studied in detail is IS1237, whose termini was found to have two active promoters, both capable of promoting transcription of adjacent genes<sup>62</sup>. Accordingly, desired genes can be efficiently expressed in *Lxc* using these promoters- especially the 3' promoter being stronger<sup>62</sup>. These findings are particularly important if the use of *L. xyli* in a paratransgenesis model to express anti-*Leishmania* molecules in the sand fly gut is to be considered.

### **Alphaproteobacteria**

Unlike *L.xyli*, the presence of *Wolbachia* spp. in the gut of laboratory reared *P. papatasi* was expected. The genus *Wolbachia* has been recognized as the most prevalent bacterial symbiont of arthropods and nematodes known to date<sup>63</sup>. In fact, the discovery of the

association of *Wolbachia* with filarial nematodes has been noted the most exciting finding in filarial research during the last decade<sup>64</sup>.

These maternally transmitted, intracellular,  $\alpha$ -proteobacteria endosymbionts renowned for inducing significant changes in their hosts' biology maintain a special - apparently contradicting- relationship with different hosts. A relationship that could be at times considered parasitic – when infecting arthropods- or mutualistic at other instances- such as with filarial nematodes<sup>63,64</sup>. *Wolbachia*-induced changes encompass a wide range of reproductive manipulations, yet all share a common goal: maintaining the vertical transmission of *Wolbachia*, sometimes even at the expense of its host<sup>65</sup>. This fact renders *Wolbachia* tremendous potential to be practically implemented to deliver transgenes to a large population<sup>66</sup>, thereby making it a significant candidate if a paratransgenesis model to control *Leishmania* is implemented.

The presence of an essential endosymbiotic *Wolbachia* in the filarial nematode *Onchocerca volvulus* that causes river blindness, and in *Brugia malayi* and *Wuchereria bancrofti* causative agents for lymphatic filariasis illustrates the significant role of endosymbiotic bacteria in the pathogenesis and therapy of parasitic diseases<sup>67</sup>. Studies have shown that targeting *Wolbachia* produces sustained anti-filarial effects, and that the elimination of *Wolbachia* by antibiotics prevents ocular damage and improves skin lesions associated with these parasitic diseases<sup>67</sup>. Accordingly, it has been suggested that *Wolbachia* present in filarial nematodes plays a role in the survival of these parasites in human hosts, most likely through affecting the hosts' immune system in ways that are yet to be understood<sup>64</sup>.

Furthermore the ability of *Wolbachia* infections to protect *Drosophila melanogaster* against RNA viruses shows that this endosymbiont also affects the immune system of its direct hosts<sup>65</sup>. These phenomena raise several questions to the role(s) of *Wolbachia* in the gut of sand flies and its effect, not only on sand flies as their direct host, but also on the pathogenesis of leishmaniasis. It is worthwhile to point out here that antibiotics such as Amphotericin B are being effectively used in the treatment of leishmaniasis and have high cure rates with the serious forms of the disease such as visceral leishmaniasis<sup>68,69</sup>.

Investigations to detect the association of *Wolbachia* with sand flies and other insects surged during the last decade after the cloning and sequencing of a rapidly evolving gene encoding *Wolbachia*'s outer surface proteins (*wsp*) by Zhou *et al.* from a large variety of insects<sup>70</sup>. It also initiated the system currently implemented in the naming and typing of the different strains of *Wolbachia* that is based on both the *wsp* gene sequences and the name of organism it was isolated from. Among other insects, this study included *Phlebotomus papatasi* sand flies collected from Israel, which were found to harbour *Wolbachia* spp. therefore their *wsp* gene sequence was designated *wpap*<sup>70</sup>.



Several studies targeting the detection of *Wolbachia* in sand flies followed. Cui *et al* was able to isolate *Wolbachia* from laboratory reared *Phlebotomus papatasi* from Israel, Egypt and Saudi Arabia, in a study covering 4 different sand fly species including 11 laboratory reared and four field caught flies<sup>71</sup>. The absence of *Wolbachia* in the majority of samples in this study led the author to conclude that *Wolbachia* infection is uncommon in sandflies<sup>71</sup>.

In a larger study covering fifteen species of sand flies, again only four species were found positive for *Wolbachia*, *P. papatasi* being among them, together with *P. perniciosus*, *L. shannoni*, *L. whitmani*<sup>72</sup>. Interestingly, despite the fact that this ratio (26%) is higher than the ratio seen with other insects (16%- 22%), this figure was considered by the author to be underestimated not truly representing association of *Wolbachia* with sand flies. This was primarily because the PCR method employed in *Wolbachia* surface protein (*wsp*) detection is prone to generation of false negatives, besides the fact that this study covered only 15 out of the 800 species of sand flies on the globe<sup>72</sup>.

The association of *Wolbachia* with the Egyptian population of sand flies was also investigated by Kassem *et al.* using the *wsp* gene primers<sup>73</sup>. Among four laboratory colonies investigated belonging to 3 different species; *Phlebotomus bergeroti*, *P. langeroni*, and two *P. papatasi* colonies, only one of the *P. papatasi* colonies obtained from Sinai was found to harbour *Wolbachia*. These findings pointed out to the possibility of the presence of both inter and intra species variation in the distribution of *Wolbachia* even among sand flies collected from the same geographical location<sup>73</sup>. Recently, *Wolbachia* was detected for the first time in sand flies from France belonging to *Phlebotomus perniciosus* and *Sergentomyia minuta* species. This was also the first time to report *Wolbachia* in *Sergentomyia minuta* species of sand flies<sup>74</sup>.

On investigating the distribution of *Wolbachia* inside the tissues of sand flies belonging to the *Phlebotomine* genus, Benlarbi *et al* found that *Wolbachia* is present both in the thorax – where the infective forms of *Leishmania* develop- as well as the abdomen where infection of reproductive tissue occurs<sup>66</sup>. Another important observation was the high densities of *Wolbachia* infections among some sand fly populations. Both these observations entitle *Wolbachia* to become an important candidate if delivering transgenes- to halt the transmission of *Leishmania* parasites- to large populations of sand flies is required<sup>66</sup>.

Another important issue that has to be addressed is the ability of *Wolbachia* to confer changes in its hosts' genomes. The exchange of genetic materials between the widespread endosymbiont *Wolbachia* and its host the adzuki bean beetle, *Callosobruchus chinensis*, has been reported<sup>75</sup>. Despite the fact that the mechanism of this gene transfer is still not understood, it is believed that the intimate association of this endosymbiont with germ line reproducing cells facilitates this exchange<sup>75</sup>. Interestingly, recent gene inserts belonging to *Wolbachia*'s genome have been located in about one third of the genomes of invertebrates and the complete *Wolbachia* genome was found in others -such as the tropical fruit fly

*Drosophila ananassae*<sup>65</sup>. Another scenario of genomic interaction between symbiotic bacteria and their hosts was detected with the symbiotic gut fauna of a vector of another parasitic disease: the tsetse fly. Significant changes have been noted in the genome of the well studied tsetse fly symbiont, *Wigglesworthia glossinidia* to enable its transition from a free living state to an endosymbiotic life style<sup>76</sup>. As the two organisms coevolved, the genome of *Wigglesworthia* experienced a huge reduction in size -amounting to about 700kb- leading to the elimination of genes that have counterparts in their host<sup>76,77</sup>.

These examples provide an explicit example of the possibility and prevalence of lateral gene transfer between endosymbiotic bacteria and their arthropod hosts. These examples also demonstrate how this relationship significantly impacts the evolution of the genomes of the host at times and the gut bacteria at others. The impact of *Wolbachia* on the genome of sand flies and the extent to which it could have influenced its capacity to harbour and transmit *Leishmania* is among the interesting issues expected to be unleashed following the sequencing of the genomes of *Phlebotomus papatasi* and *Lutzomyia longipalpis* that are currently in process.

Besides *Wolbachia* spp., the other Alphaproteobacteria identified in the gut of *P.papatasi*- yet this time by both the culture dependent and the culture independent techniques- is *Ochrobactrum* spp. The genus *Ochrobactrum* lies in close proximity to the genus *Brucella* and is known to contain highly versatile species isolated from a variety of environments<sup>78</sup>. These include *O. anthropi* as the earliest characterized species, and also known to be an opportunistic human pathogen and among the most resistant Gram negative rods<sup>78</sup>. *Ochrobactrum* species were also isolated from environments polluted with chromate and nonyl phenol, entitling them to become prospective candidates in bioremediation processes<sup>79,80</sup>. Sequencing of the genome of the type strain *O. anthropi* (ATCC 49188T) revealed the presence of a homolog of the complete *virB* operon, which was identified as the major determinant of virulence in this genus. Interestingly, this is the same operon used by *Brucella* spp. for survival and multiplication in macrophages, and that responsible for the DNA transfer processes in *Agrobacterium tumefaciens*<sup>81</sup>.

In terms of their symbiotic relationships, several species of this genus have been found in association with a variety of hosts including insects, animals, plants and even nematodes. *Ochrobactrum* spp. was among the symbiotic bacteria involved in hemicellulose degradation in the gut of termites<sup>82</sup>. In addition, *O. anthropi* was isolated from the intestinal tract of the housefly *Musca domestica* larvae<sup>83</sup>, and an *Ochrobactrum* spp. designated AK was also identified in the gut of the sand fly *P. duboscqi*<sup>24</sup>. Recently, other species- *O. lupini* and *O. cytisi*-were identified in a symbiotic relationship with legumes, functioning as a legume nodulating bacterium<sup>84,85</sup>.

*Ochrobactrum* spp. was isolated from two other interesting places. It was found with the symbiont *Photorhabdus luminescens* in nematodes belonging to genus *Heterorhabditis*.

Species of nematodes belonging to this genus are known to be entomopathogenic nematodes; being obligate parasites of insects<sup>86</sup>. The endosymbiotic bacterium of these nematodes- *Photorhabdus luminescens*- has a recognized contribution to the reproductive functions of this nematode and its pathogenicity to insects. In fact, insects are killed by toxemia and septicemia resulting from the release of the nematodes' symbiotic bacteria *Photorhabdus luminescens* into the hemolymph of the insect, after penetration of the former into the insect's body cavity<sup>86</sup>. Despite being a noteworthy finding, the exact reason behind the presence of *Ochrobactrum* spp. with this bacterial symbiont in entomopathogenic nematodes is still unknown<sup>86</sup>. Yet, this finding again draws the attention onto how endosymbiotic bacteria are implicated in the pathogenesis of conditions caused by their hosts. While the role of *Ochrobactrum* in aiding *Heterorhabditis* nematodes in killing its insect hosts is currently under investigation, it is worthwhile to investigate the role played by the same bacterium both in the sand fly- its direct host- or for the *Leishmania* parasite during the development of leishmaniasis in human hosts. It would also be useful to stand upon another finding reported lately where *O. anthropi* was found to cause cartilage degeneration and other bone deformities in cane toads<sup>87</sup>, and the possibility of this phenomenon being linked- in any way- to cartilage deformities of muco-cutaneous leishmaniasis.

### **Betaproteobacteria**

In addition to the two aforementioned Alphaproteobacteria, a third Proteobacterium was identified in the gut of laboratory reared *P. papatasi*, yet this time a Betaproteobacterium identifiable at the species level as *Achromobacter xylosoxidans*. Previously known as *Alcaligenes xylosoxidans*, this bacterium was found to possess several interesting features such as the production of class D  $\beta$ -lactamases<sup>88</sup>, the ability to degrade polychlorinated biphenyls<sup>89</sup>, and thiodiglycol (the mustard gas hydrolysis product)<sup>90</sup>, and also to act as an endophytic bacteria that improves plant growth<sup>91</sup>.

Moreover, *Achromobacter xylosoxidans* has been reported in the guts of several insects from red fire ants<sup>92</sup>, to the fruit piercing and blood sucking moths *Calyptra*<sup>93</sup>, the Hessian fly<sup>94</sup> and importantly, in the gut of mosquito vectors of malaria belonging to *Culex* and *Anopheles* spp.<sup>22,95</sup>. *Achromobacter xylosoxidans* subsp. *denitrificans* also occurs as a non pathogenic endophyte in the xylem of several plants, and was identified as a symbiotic bacterium in the gut of the xylophagous insect *Homalodisca coagulata*<sup>96</sup>. This insect is the primary vector of *Xylella fastidiosa*; a xylem colonizing bacterial pathogen causing numerous plant diseases of significant economic importance<sup>96</sup>. In an attempt to halt the spread of this plant pathogen, the ability of the symbiont *Achromobacter xylosoxidans* to deliver transgenes- to render the vector incompetent and to cure infected plants at the same time- was tested and proved successful<sup>96</sup>. Attempts on designing a successful paratransgenesis model to halt the spread of *Leishmania* can build on this finding and implement this bacterium to successfully deliver anti-*Leishmania* molecules in the sand fly guts.

## Gammaproteobacteria

While both the aforementioned alpha and beta classes of the phylum Proteobacteria were absent in the gut of field caught flies, a third class of Proteobacteria appeared to dominate. In fact, it was not surprising to find Gammaproteobacteria- which were almost entirely affiliated to the genus *Rahnella*- as the predominant bacteria in field flies. Firstly, being members of the family Enterobacteriaceae widely known to have special affinity to inhabit the gut. Secondly, because bacteria belonging to the genus *Rahnella* are abundant in nature: in the phyllosphere, rhizosphere and even in nitric acid and uranium contaminated soils<sup>97</sup>. Increased interest in *Rahnella* strains has been developing lately following the discovery of a number of remarkable features of this organism. *Rahnella* is able to contribute to plant nutrition by fixing nitrogen and supplementing plant with usable phosphate through solubilization of hydroxyapatite<sup>97</sup>. Moreover, polysaccharides such as levan and lactan that have important industrial applications are also produced by *Rahnella*<sup>97</sup>. Another *Rahnella* spp. isolated from acidic subsoil is capable of immobilizing and precipitating toxic uranium (VI) via an intrinsic phosphatases activity<sup>98</sup>.

Recently, *Rahnella* was found in the gut of river trout (*Salmo trutta fario* L.) from the Lithuanian river Skroblus in significant amounts<sup>99</sup>. The abundance of *Rahnella* in the gut of a variety of insects has been reported, placing it among the major insect intestinal flora together with *Pantoea* and *Enterobacter* which are also members of Enterobacteriaceae<sup>100</sup>. Among these insects are longicorn beetles of different types collected from several places in Korea<sup>101</sup>, the spined soldier bug *Podisus maculiventris*<sup>102</sup>, the southern pine bark beetles *Dendroctonus frontalis* Zimmermann where it occurred as the most common species<sup>103</sup>, the herbivorous *Longitarsus* flea-beetles where they represented the second most abundant intestinal species<sup>104</sup> and also the spruce bark beetle, *Ips typographus* L.<sup>105</sup>. Nevertheless, unlike with fish where *Rahnella* is involved in complex carbohydrates fermentation and nitrate reduction, the role of *Rahnella* in insect guts is still unknown<sup>104</sup>.

A study investigating the plasmids of the genus *Rahnella* revealed the presence of plasmids in about 19% of *Rahnella* isolates<sup>97</sup>. This is considered an average number when compared to members of the family Enterobacteriaceae where plasmids are at times abundant as with *Escherichia coli* - 42% of the isolates have a single plasmid at least- and *Citrobacter freundii*- where plasmids are extremely rare<sup>97</sup>. Moreover, regions of striking sequence homology were found between these plasmids and chromosomes of two other bacteria: *Photobacterium luminescens* TT01 and *Erwinia tasmaniensis* Et/99<sup>97</sup>. Taking into consideration the overlap in habitat between these bacteria and *Rahnella*, it was suggested that the presence of plasmids in *Rahnella* is significant, thereby enabling the lateral transfer of genetic elements between *Rahnella* and distinct genera<sup>97</sup>. This feature in addition to the abundance of this genus both in nature and in the guts of wild *P. papatasi* as evident in this study may entitle *Rahnella* to become a prospective candidate to be used in a paratransgenesis model. In fact, the use of *Rahnella* for this purpose had been proposed

after finding it in abundance in the gut of the Pacific wire worm *Limonius canus*- the serious potato pest<sup>106</sup>.

Nevertheless, it would be important to consider the capability of *Rahnella* to encode antibiotic resistance genes and enterotoxins<sup>97</sup>. Although reports on infections with *Rahnella* are restricted to immunocompromised patients, with quick recovery and susceptibility to antibiotics been reported<sup>97</sup>, thorough research before progressing with this step is essential.

It is important not to overlook the presence of the other Gammaproteobacterium identified in this study (*Stenotrophomonas maltophilia*) despite the fact that only a single colony was isolated in the culture dependent analysis of laboratory reared *P.papatasi*. The recently available genome of *S. maltophilia* reveals, "a startling array of antimicrobial drug resistance gene determinants"<sup>109</sup>. *S. maltophilia* is known for its abundance in nature and ability to degrade xenobiotic compounds, and is also known as an important opportunistic pathogen responsible for a considerable number of nosocomial infections<sup>110</sup>.

Perhaps among the most intriguing findings is *S. maltophilia* being reported in one of the recent studies investigating gut fauna of the sand fly *Lutzomyia longipalpis* in three areas in Brazil, two of which were endemic for visceral leishmaniasis and one from a non-endemic region<sup>17</sup>. Interestingly, among all the other bacteria isolated, *S. maltophilia* was the only gut bacterium found to be common between flies of all the three regions. Isolating the same bacterium in this study, yet this time from laboratory reared flies, on the other side of the globe, and from uninfected flies is a significant finding. In a way, it suggests that this bacterium is most likely not environmentally acquired, being present regardless of the location of the caught flies. In addition, the fact that it was isolated this time from laboratory reared flies that have just emerged, and that these flies belong to a different genera of *Leishmania* transmitting sand flies could suggest a symbiotic association between this bacterium and sand flies in general not just *P.papatasi*. The small number of wild flies analysed in this study possibly hindered its isolation from wild *P.papatasi*, thereby necessitating further investigation before confirmation of this symbiotic relationship. It would be equally important to investigate how the intrinsic drug resistance of this bacterium could be affecting both sand flies and *Leishmania*. Indeed it is intriguing to speculate that the increasing insecticidal resistance exhibited by the flies and the emerging antimicrobial resistance of the *Leishmania* parasites could be linked in a way or another to interactions with this bacterium.

## **Bacilli**

Finally, it would be important to draw attention to bacteria belonging to this class, being numerous reported in previous literature dealing with sand fly gut fauna, and also appearing in abundance in field flies investigated here. The genus *Bacillus* combines two important features: being the most abundant genus found in insect guts<sup>107</sup>, as well as including many strains pathogenic to insects<sup>108</sup>. Accordingly, it has been referred to as the

most important genus producing microbial pesticides<sup>108</sup>. *B.mycoides*, *B.subtilis*, *B.mesentericus*, *B.ceres*, *B.thruingiensis*, *B.niacini*, *B.megaterium* and *B.pumilus* are species that have been reported to occur in the gut of sand flies<sup>6,40,43</sup>. Nevertheless, none of these species were identical to the ones encountered in this study. This lack of overlap in the species of *Bacillus* isolated most likely reflects the differences in the environments from which the field flies were collected. For example, *Bacillus megaterium* that is present in biofertilizers widely used in the state of Bihar, India, was isolated from the guts of a number of sand flies inhabiting that area<sup>6</sup>. The hypothesis that the *Bacillus* species are picked by sand flies from the environment may be strengthened by the fact that none of the 100 newly emerged lab reared *P. papatasi* included in this study were found to harbour any members of this genus. This observation also extends to members of the naturally abundant genus *Rahnella* found in high numbers only in field caught flies and completely absent in laboratory flies. Accordingly, the colonization of these bacteria to the gut of field caught flies should be regarded taking into consideration the natural abundance of bacteria from these genera. In other words, caution should be practiced before regarding these bacteria to be in a symbiotic relationship with sand flies.

## CONCLUSION

Combined, the results of both culture dependent and independent methods identified four bacterial phyla, and 26 species, of bacteria in the guts of laboratory reared *P.papatasi* and field caught sand flies. The Phylum Actinobacteria represented by *Leifsonia* spp. predominated laboratory isolates, whereas members of the Proteobacteria phylum were shared between laboratory and field caught sand flies. The premise that culture independent approaches would identify more species was supported by the data; roughly double the number of bacterial species was identified in laboratory reared flies using culture independent approach, despite using a smaller sample. Although culture independent techniques may fail to fully characterize the community assemblage (e.g.; very rare species may not amplify sufficiently to detect in the clone library), this approach is superior to culture dependent methods. Because of advances by this study in the optimization of the molecular based approach, future studies of sand fly gut fauna will be relatively easy to conduct.

Some of the bacterial species identified in this study have been genetically modified in previous studies and may therefore serve as promising candidates in a paratransgenesis model to halt the spread of *Leishmania*. Furthermore, some species are reported to inhabit the gut of sand flies elsewhere, which could aid in identifying possible symbiotic associations between sand flies and bacteria. Of course, this study also identified bacteria which have not been previously characterized in the sand fly gut. Further work is required for a complete understanding of their function(s) in the gut and the ways in which they may influence biological processes of the vector, the life cycle of the parasite or even disease establishment in hosts.

The detailed characterization of the bacterial gut fauna of sand flies and the role(s) they play should no longer remain a road less travelled, or travelled solely for locating bacterial candidates for paratransgenesis. Data from this study serves as a platform for more comprehensive studies addressing these organisms that occupy the tiny gut space of sand flies with the *Leishmania* parasites. Such studies hold strong potential to revolutionize the entire way we look upon leishmaniasis: its pathogenesis, transmission and control.

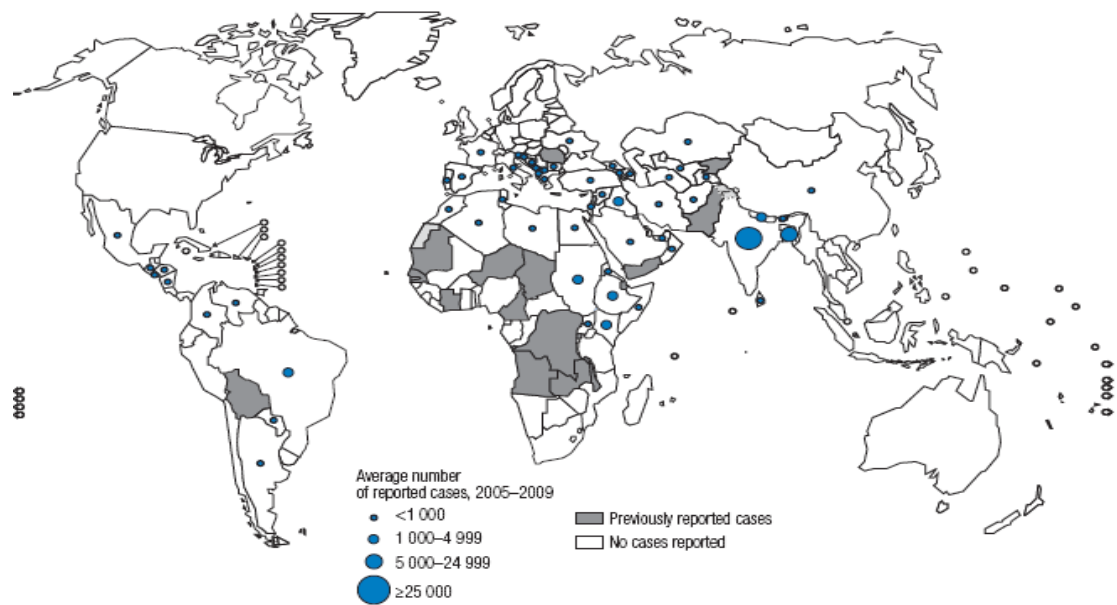


Figure 1: Distribution of visceral leishmaniasis, worldwide, 2009 (WHO: Working to overcome the global impact of neglected tropical diseases.)<sup>4</sup>

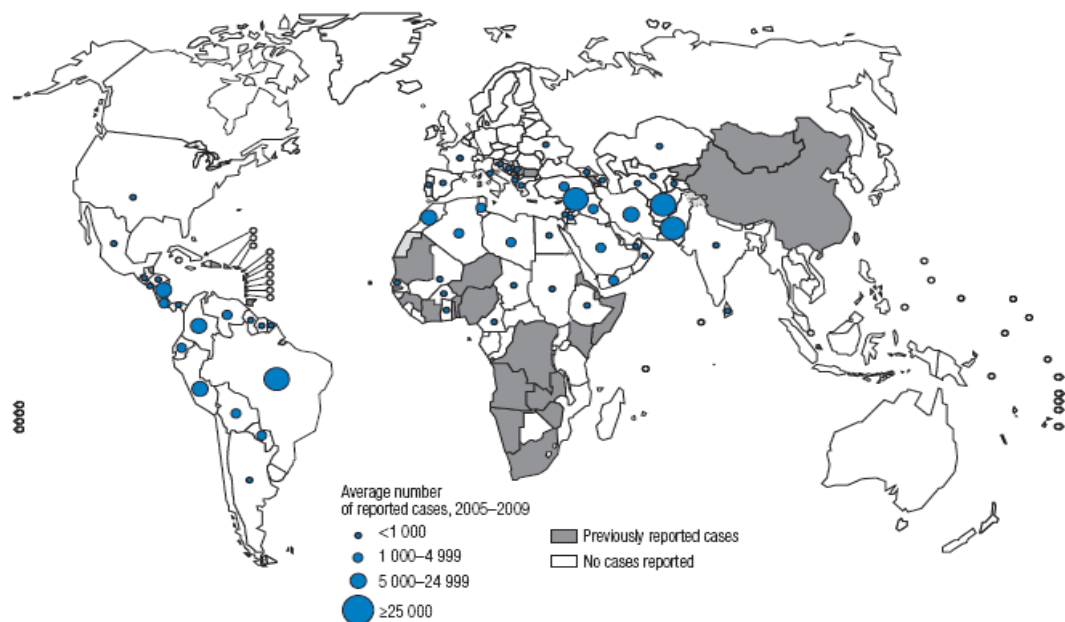


Figure 2: Distribution of cutaneous leishmaniasis, worldwide, 2009 (WHO: Working to overcome the global impact of neglected tropical diseases.)<sup>4</sup>



Table 1: Preliminary assessment of the isolated pure colonies from culture dependent analysis of laboratory reared *P. papatasi*

Initials of pool	Colonies isolated	Initial characterization of colonies						
		Gram reaction	Size	Pigmentation	Margin	MacConkey Agar	Oxidase Test	Urease Test
V	V1	G+ve	tiny	creamy	entire	no growth	+ve	-ve
	V2	G-ve	small	white	entire	growth (lactose non-fermenter)	+ve	+ve
	V3	G-ve	medium	green	entire	growth (lactose non-fermenter)	-ve	-ve
	V4	G+ve	large	creamy	entire	no growth	-ve	-ve
	V5	G-ve	smaller	dark yellow	entire	no growth	+ve	-ve
D	D1	G-ve	small	white	entire	growth (lactose non fermenter)	+ve	+ve
ND1	M1	G+ve	tiny	creamy	entire	no growth	+ve	-ve
ND2	M2a	G+ve	tiny	creamy	entire	no growth	+ve	-ve
	M2b	G+ve	tiny	creamy	entire	no growth	+ve	-ve
	M2c	G-ve	small	white	entire	growth (lactose non-fermenter)	+ve	+ve
ND3	M3a	G+ve	tiny	creamy	entire	no growth	+ve	-ve
	M3b	G-ve	small	white	entire	growth (lactose non-fermenter)	+ve	+ve
ND4	M4a	G+ve	tiny	creamy	entire	no growth	+ve	-ve
	M4b	G-ve	small	white	entire	growth (lactose non-fermenter)	+ve	+ve

Table 2: Assembly groups from culture dependent analysis of laboratory reared *P. papatasi*

Group Name	Isolate Initials
Assembly_1	D1, V2, M3b, M2c, M4b
Assembly_2	M2a, M2b, M1, M4a, M3a, V1

16S rRNA gene sequences of bacterial isolates recovered from the plate cultures were assembled thereby grouping identical isolates together. Two groups resulted; the first contained five identical isolates, and the second group contained six identical.

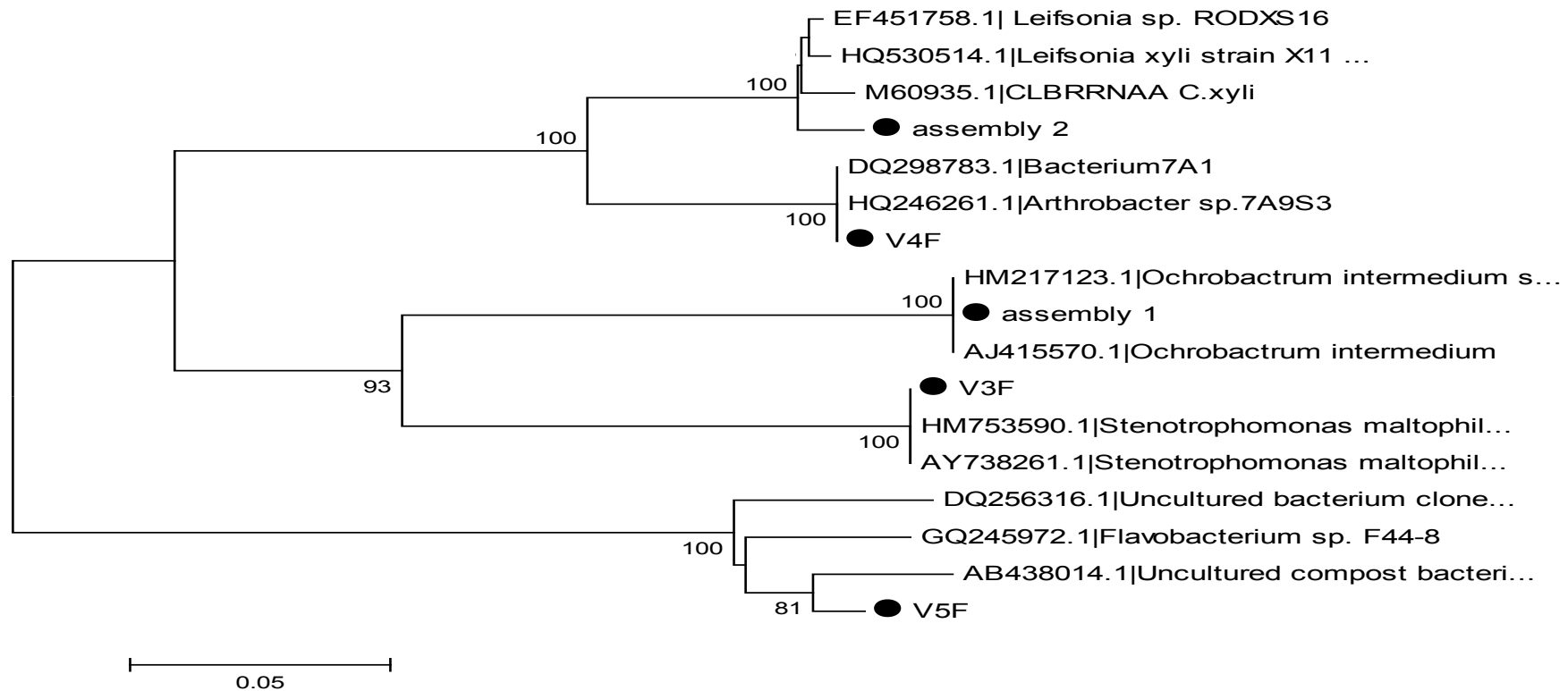
**Table 3: Diversity of gut bacteria of laboratory reared *P. papatasi* (Culture Dependent Analysis)**

Identity of DNA sequence/ group (no. of isolates in this group)	Accession no. of closest relative according to NCBI Blast	Name of closest relative according to NCBI Blast for the 16S rRNA gene	Similarity score %
Phylum Actinobacteria,Class Actinobacteria ,Subclass Actinobacteridae , Order Actinomycetales, Suborder Micrococcineae , Family Microbacteriaceae			
V4F	HQ246261.1	Arthrobacter sp. 7A9S3	100%
assembly_2 (6)	EF451758.1	Leifsonia sp. RODXS16	98%
Phylum Bacteroidetes, Class Flavobacteria , Order Flavobacteriales, Family Flavobacteriaceae			
V5F	AB438014.1	Uncultured compost bacterium gene	93%
Phylum Proteobacteria, Class Alphaproteobacteria ,Order Rhizobiales , Family Brucellaceae			
assembly_1 (5)	HM217123.1	Ochrobactrum intermedium strain DSQ5	100%
Phylum Proteobacteria, Class Gammaproteobacteria, Order Xanthomonadales , Family Xanthomonadaceae			
V3F	HM753590.1	Stenotrophomonas maltophilia	100%

PHYLA

Phylum	Relative Abundance (%)
Actinobacteria	50%
Proteobacteria	43%
Bacteroidetes	7%

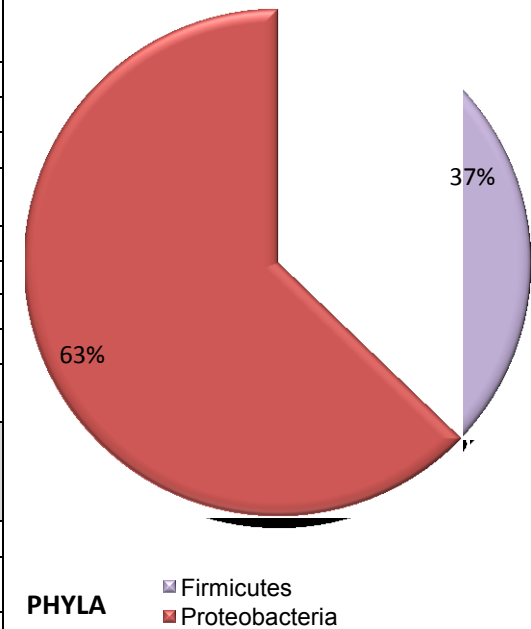
The table above displays the 16S rRNA gene sequences obtained from plate cultures classified according to the closest match in the GenBank database. The chart illustrates the relative abundance of the identified phyla.



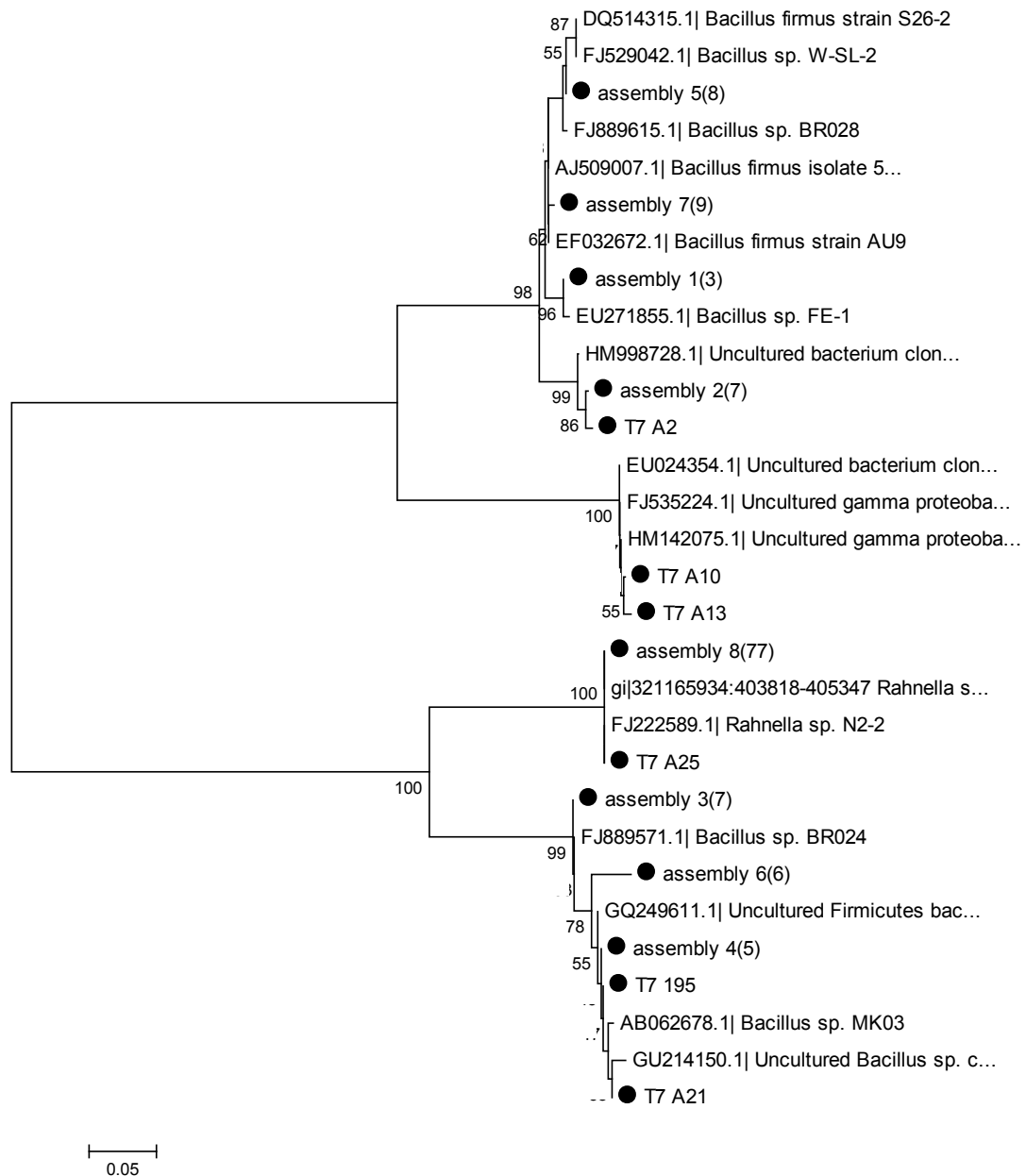
**Figure 3: Phylogenetic affiliations of the bacteria isolated from laboratory reared *P. papatasi* using culture dependent technique.** The black circles represent species isolated in this study and the rest are the highest matching species downloaded from RDP and GenBank. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Numbers at the nodes indicate percent bootstrap values above 50 (1000 replicates).

**Table 4: Diversity of gut bacteria of field caught sand flies (Culture Dependent Analysis)**

Identity of DNA sequence/ group (no. of isolates in this group)	Accession no. of closest relative according to NCBI Blast	Name of closest relative according to NCBI Blast for the 16S rRNA gene	Similarity score %
<b>Phylum Firmicutes , Class Bacilli , Order Bacillales, Family Bacillaceae</b>			
assembly_6 (6)	EU271855.1	<i>Bacillus sp.</i> FE-1	100%
assembly_1 (3)			99%
assembly_3 (7)	FJ889571.1	<i>Bacillus sp.</i> BR024	99%
assembly_5 (8)	FJ889615.1	<i>Bacillus sp.</i> BR028	99%
assembly_4 (5)	GU214150.1	Uncultured <i>Bacillus sp.</i> clone IAFpp7230	99%
assembly_2 (7)	AB062678.1	<i>Bacillus sp.</i> MK03	98%
assembly_7 (9)	EF032672.1	<i>Bacillus firmus strain</i> AU9	98%
T7_195	GQ249611.1	Uncultured Firmicutes	95%
T7_A21	AB062678.1	<i>Bacillus sp.</i> MK03	95%
T7_A2	HM998728.1	Uncultured bacterium clone 98B-1_G07_T3	94%
<b>Phylum Proteobacteria , Class Gammaproteobacteria , Order Enterobacteriales, Family Enterobacteriaceae</b>			
assembly_8 (77)	FJ222589.1	<i>Rahnella sp.</i> N2-2	99%
T7_A13	HM142075.1	Uncultured gamma proteobacterium clone CS11	97%
T7_A10			96%
T7_A25	CP002505.1	<i>Rahnella sp.</i> Y9602	95%



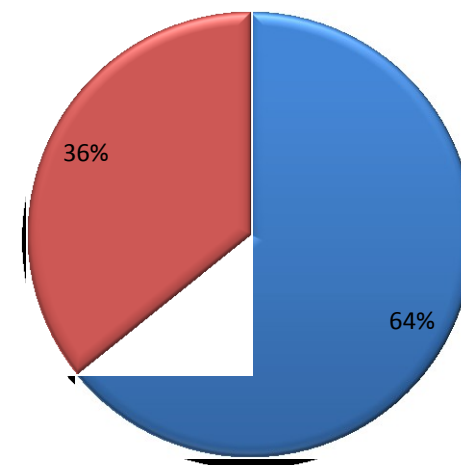
The table above displays the 16S rRNA gene sequences of the cloned isolates of bacteria recovered from wild fly guts classified according to the closest match in the GenBank database. The chart illustrates the relative abundance of the identified phyla.



**Figure 4: Phylogenetic affiliations of the gut bacteria isolated from field caught sand flies using culture dependent techniques.** The black circles represent species isolated in this study and the rest are the highest matching species downloaded from RDP and GenBank. The numbers in brackets refer to the number of isolates present in this group. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Numbers at the nodes indicate percent bootstrap values above 50 (1000 replicates).

**Table 5: Diversity of gut bacteria of laboratory reared *P. papatasi* (Culture Independent Analysis)**

Identity of DNA sequence/ group (no. of isolates in this group)	Accession no. of closest relative according to NCBI Blast	Name of closest relative according to NCBI Blast for the 16S rRNA gene	Similarity score %
<b>Phylum Actinobacteria, Class Actinobacteria , Subclass Actinobacteridae , Order Actinomycetales , Suborder Micrococcineae , Family Microbacteriaceae</b>			
Assembly_6 (20)	DQ232614.2	<i>Leifsonia shinshuensis</i>	99%
Assembly_7 (28)	HQ530514.1	<i>Leifsonia xyli</i> strain X11	98%
Assembly_1 (2)	AB177251.1	Uncultured bacterium gene	97%
T7_101	FJ189782.1	<i>Leifsonia xyli</i> strain CSB05	95%
<b>Phylum Proteobacteria, Class Alphaproteobacteria , Order Rickettsiales , Family Anaplasmataceae</b>			
Assembly_5 (13)	AY007547	<i>Wolbachia sp.</i> Dlem16SWol	99%
T7_A31	DQ981315.1	Uncultured bacterium clone thom i20	96%
<b>Phylum Proteobacteria, Class Alphaproteobacteria ,Order Rhizobiales , Family Brucellaceae</b>			
T7_151	FJ658472.1	Uncultured bacterium clone Winter_MachineA&B	100%
Assembly_3 (4)	FN645728.1	<i>Ochrobactrum sp.</i> KD2009-45	99%
<b>Phylum Proteobacteria, Class Betaproteobacteria , Order Burkholderiales , Family Alcaligenaceae</b>			
Assembly_4 (6)	GU586301.1	<i>Achromobacter xylosoxidans</i> strain IR-826	99%
Assembly_2 (4)	EU006066.1	<i>Achromobacter xylosoxidans</i>	99%

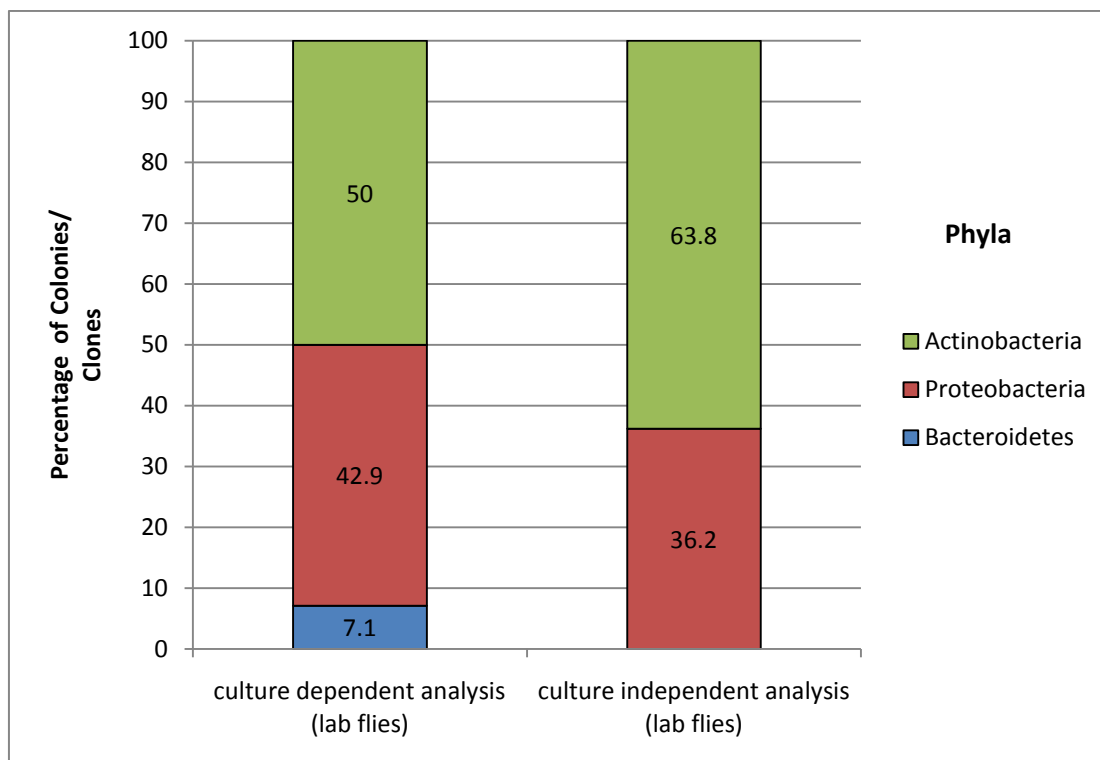


**PHYLA**  
■ Actinobacteria  
■ Proteobacteria

The table above displays the 16S rRNA gene sequences of the cloned isolates of bacteria recovered from guts of laboratory reared flies- analysed culture independently- classified according to the closest match in the GenBank database. The chart illustrates the relative abundance of the identified phyla.



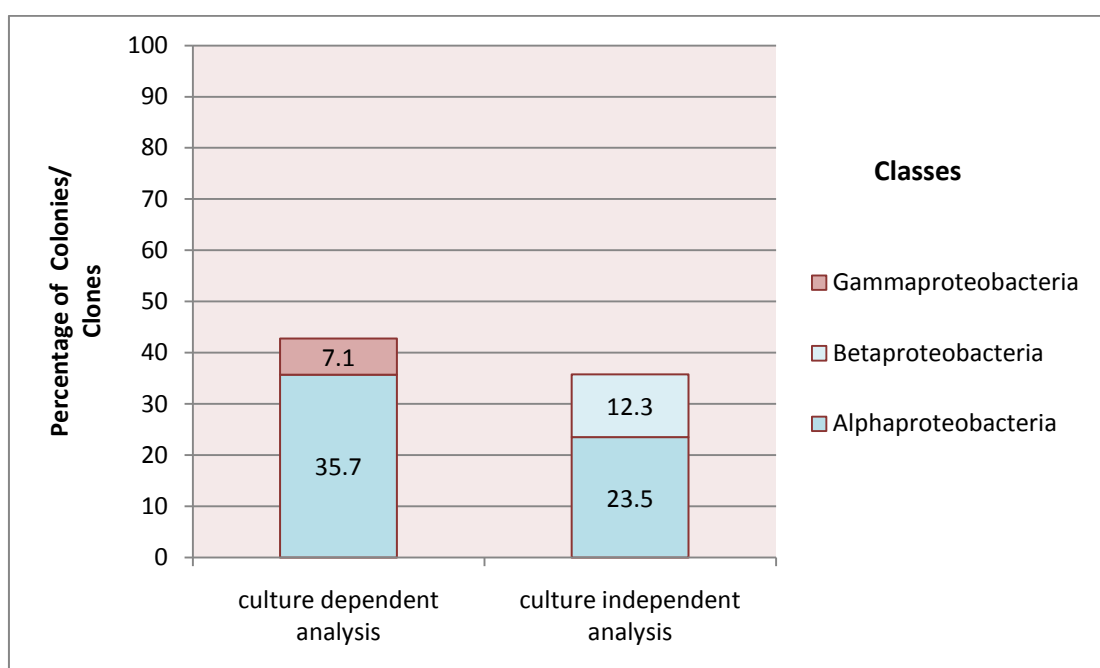
**Figure 5: Phylogenetic affiliations of the bacteria isolated from laboratory reared *P. papatasi* using culture independent techniques.** The black circles represent species isolated in this study and the rest are the highest matching species downloaded from RDP and GenBank. The numbers in brackets refer to the number of isolates present in this group. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Numbers at the nodes indicate percent bootstrap values above 50 (1000 replicates).



**Figure 6: Variation in the bacterial phyla recovered by culture dependent and culture independent approaches.**

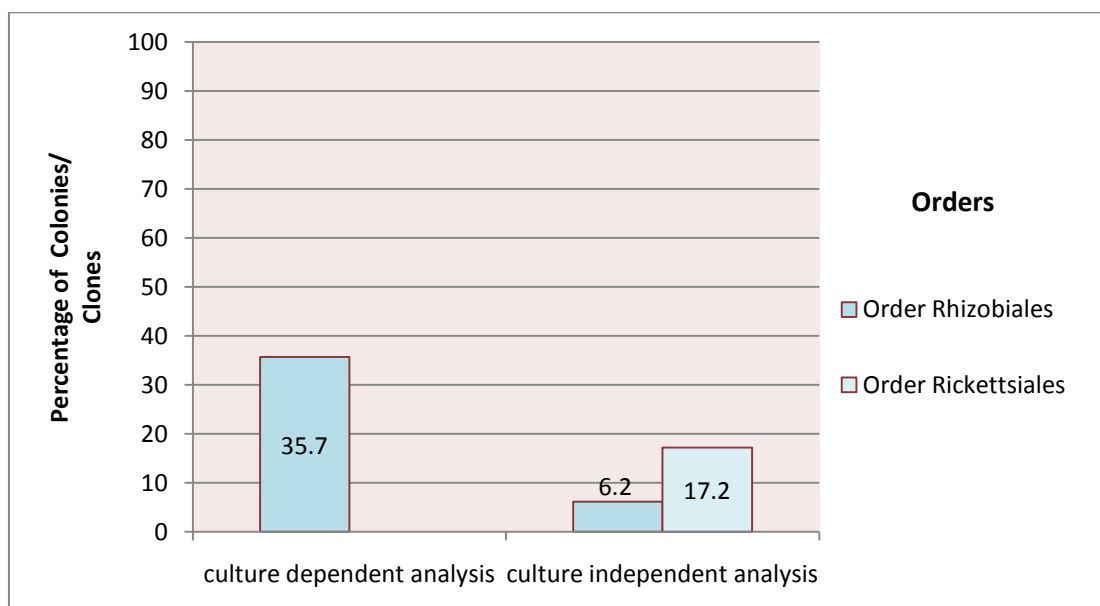
This is a generalized overview of the bacterial phyla detected in the gut of laboratory reared *P. papatasi* by both methods. However, to be able to assess the efficiency of both methods, an in depth analysis of the phyla recovered would be essential.





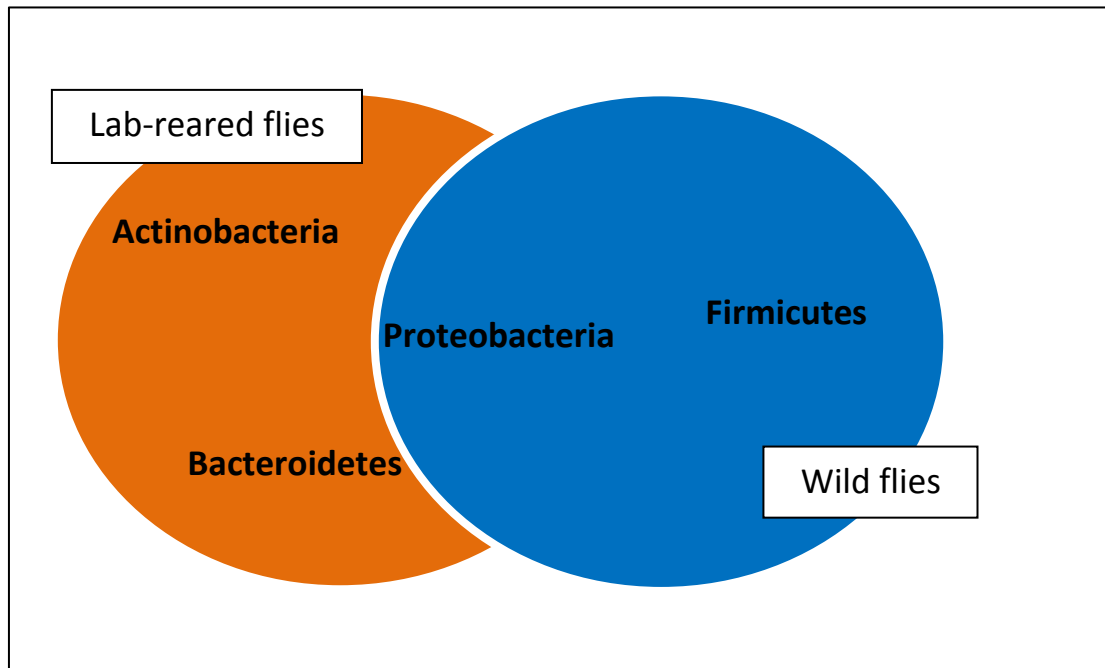
**Figure 7: Variation within the classes of Proteobacteria recovered by the culture dependent and the culture independent techniques.**

The single Gammaproteobacterium recovered was *Stenotrophomonas maltophilia*, the Betaproteobacteria affiliated isolates comprised of six *Achromobacter xylosoxidans* strain IR-826 isolates and four *Achromobacter xylosoxidans* isolates. The Alphaproteobacteria isolates recovered varied between the two methods and are depicted in the figure below.



**Figure 8: Orders of Alphaproteobacteria identified using culture independent and culture dependent techniques.**

The Alphaproteobacteria isolates recovered by culturing included five *Ochrobactrum intermedium* strain DSQ5 isolates while the culture independent analysis recovered 13 *Wolbachia* sp. Dlem16SWol isolates, four *Ochrobactrum* sp. KD2009-45 isolates and two uncultured bacterium clones.



**Figure 9: Bacterial phyla from laboratory reared and field caught flies.**

Bacteria affiliated with the Proteobacterium phyla present the most suitable candidates for paratransgenesis owing to their presence and abundance both in field flies and lab reared flies and also because of the variation in the classes of Proteobacteria recovered from their guts.

Table 6: Bacterial species recovered from the gut of all sand flies investigated in this study using culture dependent and culture independent techniques

Bacterial Class	Identified species	Presence in lab flies using culture dependent analysis (Accession. No)	Presence in lab flies using culture independent analysis (Accession. No)	Presence in field caught flies using culture dependent analysis (Accession. No)	Previous reports in sand fly guts
ACTINOBACTERIA	<i>Leifsonia</i> spp.	(EF451758.1)	(DQ232614.2, HQ530514.1, FJ189782.1)	ND	-
	<i>Arthrobacter</i> spp.	(HQ246261.1)	ND	ND	-
	Uncultured bacterium gene	ND	(AB177251.1)	ND	-
FLAVOBACTERIA	Uncultured compost bacterium gene	(AB438014.1)	ND	ND	-
ALPHAPROTEOBACTERIA	<i>Ochrobactrum</i> spp.	(HM217123.1)	(FN645728.1)	ND	(Volf <i>et al.</i> , 2002) <sup>24</sup>
	<i>Wolbachia</i> spp.	ND	(AY007547)	ND	(Benlarbi <i>et al.</i> , 2003) <sup>63</sup> (Zhou <i>et al.</i> , 1998) <sup>67</sup> (Cui <i>et al.</i> , 1999) <sup>68</sup> (Ono <i>et al.</i> , 2001) <sup>69</sup> (Kassem <i>et al.</i> , 2003) <sup>70</sup> (Matsumoto <i>et al.</i> , 2008) <sup>71</sup>
	Uncultured bacterium clone	ND	(DQ981315.1, FJ658472.1)	ND	-
BETAPROTEOBACTERIA	<i>Achromobacter xylosoxidans</i>	ND	(GU586301.1, EU006066.1)	ND	-
GAMMAPROTEOBACTERIA	<i>Stenotrophomonas maltophilia</i>	(HM753590.1)	ND	ND	(Hillesland <i>et al.</i> , 2008) <sup>6</sup> (Gouveia <i>et al.</i> , 2008) <sup>17</sup> (Oliveira <i>et al.</i> , 2000) <sup>43</sup>
	<i>Rahnella</i> spp.	ND	ND	(FJ222589.1, CP002505.1)	-
	Uncultured bacterium clone	ND	ND	(HM142075.1)	-
BACILLI	<i>Bacillus</i> spp.	ND	ND	(EU271855.1, FJ889571.1, FJ889615.1, AB062678.1, EF032672.1, AB062678.1)	(Hillesland <i>et al.</i> , 2008) <sup>6</sup> (Rajendran <i>et al.</i> , 1982) <sup>40</sup> (Oliveira <i>et al.</i> , 2000) <sup>43</sup>
	Uncultured bacterium	ND	ND	(HM998728.1, GQ24961.1, GU214150.1)	-

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